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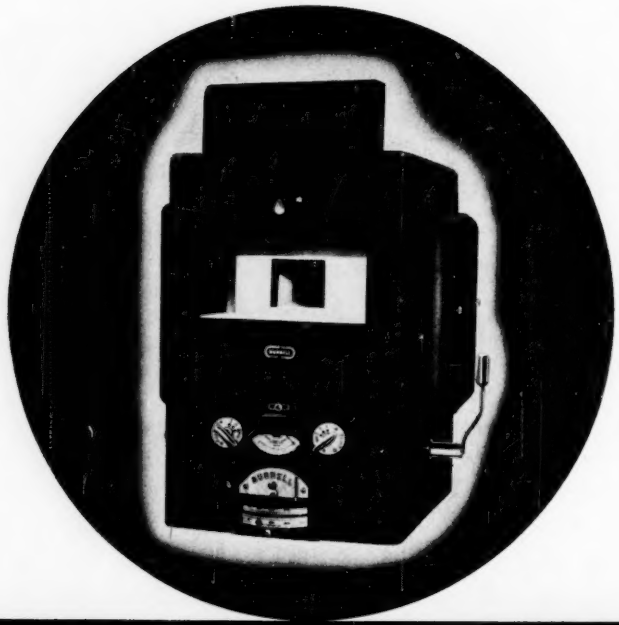

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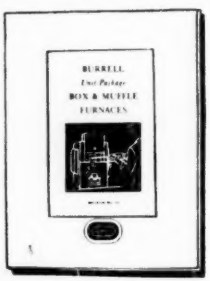

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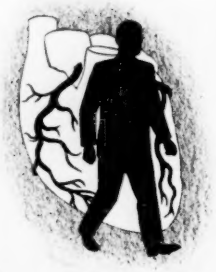

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As in the past, the Editors' principal goal is the maintenance of highest editorial standards. In this effort, the contributions of the Editorial Committee and of the reviewers listed below were of inestimable value.

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The Editors gratefully acknowledge the valuable contributions of the above-mentioned reviewers and those of the Editorial Committee.

THE EDITORS

THE NATURE OF THE CIRCULATING THYROID HORMONE IN GRAVES' DISEASE [1]

By I. N. ROSENBERG [2]

(From the New England Center Hospital and the Department of Medicine, Tufts College Medical School, Boston)

(Submitted for publication July 13, 1950; accepted, October 2, 1950)

Numerous clinical investigations have demonstrated that the concentration of plasma protein-bound iodine or serum precipitable iodine is a reliable index of thyroid gland activity, the values correlating well with the basal heat production (1) and with clinical evaluation of thyroid function (2). In myxedema the concentration is below the euthyroid range and in hyperthyroidism abnormally high values are found (3) which revert to normal following treatment of the disease by surgery (4) or by antithyroid drugs (5, 6). The serum concentration of protein-bound radioactive iodine in patients with hyperthyroidism has been found to be significantly higher than in euthyroid individuals 24 or more hours after an oral dose of I^{131} (7, 8). Similar observations have been made in animal studies; rats receiving thyrotropin injections show plasma concentrations of protein-bound I^{127}, and, if injected with tracer I^{131}, of protein-bound I^{131} which are higher than normal controls, while in thyroidectomized animals the corresponding figures for protein-bound iodine in both isotope forms are very low (9).

The chemical nature of the plasma protein-bound iodine in normal man has been the subject of several studies. Lerman (10) demonstrated by serological methods that human blood contains no detectable thyroglobulin. Trevorrow (11) reported that all the iodine in plasma may be extracted free of protein by acetone, ethanol or butanol, and subsequent application to human plasma (12, 13) and to plasma previously subjected to peptic digestion (14) of the Leland and Foster (15) method for separation of thyroxine from iodide and diiodotyrosine, showed that 70 to 90 per cent of the total iodine has solubility properties similar to thyroxine in being extractable by *n*-butanol and not re-extractable by alkali. Butanol fractionation of whole blood of euthyroid individuals following prolonged alkaline hydrolysis has led to the recovery of 40 to 60 per cent of the organic iodine in the thyroxine-like fraction (16), but these values are presumably too low since the hydrolysis is destructive of considerable thyroxine (12, 15). Thyroxine added to plasma becomes non-dialyzable (11, 12), is quantitatively precipitated with the proteins (3, 11, 12), and can be largely recovered by butanol extraction (11, 12); added diiodotyrosine is only partially recovered in the protein precipitate (3) and is extracted from butanol by base (12).

Studies on the plasma iodine of normal animals have yielded more definite evidence regarding its nature than have corresponding human investigations. Taurog and Chaikoff (12) showed that 84 per cent of the plasma iodine, and 66 per cent of the I^{131} of rats previously injected with radioactive iodine, can be recovered in the alkali-washed butanol extract, that thyroxine added to the extract containing I^{131} retains constant specific activity after repeated crystallizations and that the distribution coefficient of the radioactivity between immiscible solvents equals that of thyroxine, evidence very suggestive of the identity of thyroxine and a large fraction of the plasma iodine. Recent studies employing radioautography of paper chromatograms of butanol extracts of plasma from rats previously (24–48 hours) injected with radioactive iodine have confirmed this view. Laidlaw (17) observed spots corresponding to thyroxine and iodide as the sole radioactive components of plasma, the thyroxine constituting approximately 80 per cent of the total protein-bound I^{131}. Taurog and his associates (18) also concluded that thyroxine and iodide were the chief components. Gross and his co-workers (19) observed activity predominantly at the area corre-

[1] Aided in part by grants to Dr. E. B. Astwood from the Committee on Endocrinology of the National Research Council and from the American Cyanamid Company.

[2] U. S. Public Health Service Postdoctoral Research Fellow.

sponding to thyroxine, with very low activity at the diiodotyrosine and monoiodotyrosine spots; in addition, direct chromatography of plasma indicated that the radioactivity travelled with the proteins. The evidence derived from these animal studies strongly suggests that the circulating thyroid hormone is thyroxine in loose, non-peptide association with the plasma proteins.

Salter and his associates have investigated the nature of the protein-bound iodine in patients with myxedema and hyperthyroidism. In euthyroid and hyperthyroid individuals, butanol fractionation of plasma following peptic hydrolysis revealed that the iodine which was thyroxine-like in its solubility constituted approximately 70 per cent of the total protein-bound iodine, somewhat higher values being encountered in severe hyperthyroidism. In myxedema the thyroxine-like iodine was found to be only a small fraction (20 per cent) of the protein-bound iodine. The non-thyroxine iodine was considered diiodotyrosine-like (14). More recently, Salter and Johnston (20), on the basis of results obtained on adding thyroxine to the serum of patients, have questioned whether the circulating thyroid hormone in man is free thyroxine. At acid pH, thyroxine added to the sera of euthyroid and myxedematous subjects was only partially precipitated with the proteins by acetone, but acetone treatment of the acidified serum of a hyperthyroid patient resulted in quantitative precipitation of the organic iodine. It was inferred that the circulating hormone in hyperthyroidism is not free thyroxine.

The present work is an investigation by partition chromatographic methods of the nature of the plasma protein-bound iodine in patients with Graves' disease.

METHODS

Studies were conducted on six hyperthyroid patients who received therapeutic doses (10 to 15 millicuries each) of carrier-free radioactive sodium iodide (I^{131}). All of the patients had been previously treated with propylthiouracil or other antithyroid drugs; the administration of the antithyroid medication had been discontinued not less than three days prior to the administration of the radioiodine, and in some cases its use was resumed 48 hours after the therapeutic dose. Samples of venous blood were obtained from each patient at time intervals ranging between 48 and 240 hours after the ingestion of the isotope; the blood was placed in a centrifuge tube containing a few milligrams of dry heparin and immediately centrifuged. The plasma was drawn off, and a 1.0 cc. aliquot pipetted into a standard vial for measurement of the total content of I^{131}. The activity of this, and all other liquid samples, was estimated by means of a lead-shielded Sylvania gamma-ray counting tube, as described by Raben (21), connected with a scaling circuit of conventional type. Corrections for variations in sample volume were made by reference to a calibration curve referring counts in volumes ranging between 0.2 and 3.0 cc. to the standard volume 1.0 cc.

Extraction procedure: The procedure devised for extraction of the plasma organic iodine consisted of trichloroacetic acid precipitation of the proteins (9) and butanol extraction (22) of the acid aqueous suspension of the washed precipitate. To 2.0 cc. plasma in a 12 cc. graduated centrifuge tube was added, with stirring, 0.6 cc. ice-cold 40 per cent trichloroacetic acid. The mixture was centrifuged and the precipitate washed twice with 3–5 cc. of cold 5 per cent trichloroacetic acid, centrifuging each time and removing the liquid phase as completely as possible by means of a rubber-bulb pipette terminating in a finely drawn tip. The original supernatant liquid and the washings were combined, the volume noted, and the radioactivity of a suitable aliquot determined. The precipitate was suspended in 1 cc. of water, a few drops of 2 N NaOH added, and the pH adjusted to approximately 3 by adding 2 N HCl drop by drop. The suspension was then successively extracted three or four times by shaking vigorously each time with a 2 cc. portion of *n*-butanol saturated with water. The phases were separated by centrifuging. The water-saturated solvent was used in order to prevent the reduction in volume of the aqueous phase that otherwise would occur. The combined butanol extracts were then washed by shaking with 3 cc. of water acidified with 4 or 5 drops 4 N HCl, a pinch of NaCl crystals was added to reduce the formation of emulsion, and the butanol layer, which had a pale green color, was separated by centrifugation. The volumes of the butanol and aqueous phases were measured and the radioactivity of appropriate aliquots of each determined. When larger volumes of plasma were used, the procedure was scaled up by the use of proportionately larger volumes of reagents. The butanol extract was evaporated to dryness in vacuum at room temperature and the residue dissolved in 0.5 cc. of a solution of butanol: acetic acid: water of composition 70 : 15 : 15, respectively, by volume.[3] The solution was cooled in ice to induce separation of excess salt and centrifuged; this solution was employed in the subsequent chromatographic studies.

In several experiments, in addition to the procedure described above, direct plasma extraction with butanol

[3] In order to keep the volume of the final solution as small as possible and thus facilitate the subsequent chromatographic procedures, quantitative recovery in the butanol-acetic acid-water solution of all the I^{131} in the residue was not generally attempted. In one experiment a solvent volume of 1.0 cc. in three divided portions achieved recovery of 96 per cent of the radioactivity in the original butanol extract.

was done, the extraction being performed with five successive portions, each of twice the volume of the plasma sample. The combined butanol fractions were evaporated to dryness and the residue taken up in the manner described.

Paper chromatography: A modification of the ascending one-dimensional technique of Williams and Kirby (23) was used. The plasma extract was applied to the paper from a capillary tube so as to produce a spot about 5 mm. in diameter, replicate spots being applied 6 cm. apart along a pencil line 3 cm. above and parallel to the bottom of a rectangular sheet of Whatman No. 1 Filter paper 40 cm. long and 25 cm. wide. The spots were circled in pencil, dried under a fine stream of air at room temperature, and the solutions re-applied to the same spots, the procedure being repeated several times to increase the amount of material applied to the paper. To one spot was added 5–10 cu. mm. of a solution in butanol-acetic acid containing synthetic crystalline thyroxine (Roche-Organon), 3,5-diiodotyrosine (Hoffmann-LaRoche), 3-monoiodo-l-tyrosine (prepared by the method of Harington and Pitt-Rivers [24]), and potassium iodide; the quantity of each organic carrier added was of the order of 0.1 micromole. In several experiments, N-acetyl-l-thyroxine [4] was added as a carrier with the other components. Each large sheet of paper contained two duplicate plasma extract spots, without added carrier, a third such spot with added carrier and a fourth spot of the carrier solution alone. The paper was creased longitudinally in the center so that the sides formed an angle of about 60° and placed upright in a Pyrex cylinder (18 inches tall and 6 inches in diameter) containing 100 cc. of solvent, covered with a closely-fitting plate glass lid, and allowed to remain in the jar at room temperature for 16–20 hours, at which time the solvent front had ascended 25–30 cm. The solvent found most suitable was *n*-butanol: glacial acetic acid: water in 75:10:15 proportion by volume. Several chromatograms were also developed with the solvent *n*-pentanol: pyridine: H_2O, 40:40:15. When the solvent front had ascended to the proper height, the paper was removed, and, after the solvent front had been marked in pencil, was air dried in a hood at room temperature, cut into longitudinal strips 6 cm. wide with the origin of each spot equidistant from the long edges, and each strip marked off in centimeters from the origin to the line of solvent front. The paper was then drawn through a folded lead shield equipped with a rectangular slit 1 cm. wide and 5 cm. long, fixed immediately beneath a thin-windowed Geiger-Muller tube, so that the radioactivity of each 1 cm. length of paper could be determined. The papers were then sprayed with ninhydrin (0.25 per cent solution in water-saturated butanol) and the color developed by heating in an oven at 100° for several minutes. Acetylthyroxine, which gave no color with ninhydrin, was located by streaking the paper first with a concentrated aqueous solution of freshly prepared diazotized sulfanilic acid and then with 2.5 per cent sodium carbonate solution (25); a fading red color was produced. Iodide carrier was detected by streaking with silver nitrate solution. All R_F values (26) were obtained by measuring from the origin to the linear center of radioactivity or of color.

Radioautographs: Two-dimensional chromatograms of plasma extracts from two patients with added carrier thyroxine, diiodotyrosine and monoiodotyrosine were prepared by the ascending method (23), developing with butanol-acetic solvent in one direction and *n*-pentanol-pyridine in the other. After drying, the paper was placed directly on a sheet of no-screen X-Ray Film; film and paper were aligned by punching holes through both in opposite corners, and allowed to remain in a paper exposure holder for three to five days, when the film was developed and the paper sprayed with ninhydrin.

Chromatography on cellulose columns: The use of cellulose columns has been found helpful in the resolution of substances labelled with I^{131} (27). Columns of cellulose (Solka-Floc BW-200) 50 cm. high and 1.2 cm. in diameter were prepared by packing under a pressure of 50 cm. of mercury and equilibrating with 75:10:15 butanol: acetic acid: water solvent until 3–5 column volumes had passed through (28). A 0.3 cc. aliquot of a butanol plasma extract was added to the column, washed in with two 0.5 cc. portions of solvent and the column allowed to drip under a pressure head of 3 cm. solvent. After collection of a 20 cc. forefraction, effluent fractions each of 0.5 cc. volume were collected in flat-bottomed test tubes of uniform diameter, an automatic fraction collector operating on a constant time interval basis being used for this purpose. The radioactivity of the effluent liquid fractions was determined in the collecting tube by gamma-ray counting, as described above, without need for transfer. The contents of the three tubes containing the peak of radioactivity were evaporated to dryness in vacuum, taken up in a small volume of solvent, carrier thyroxine added, and chromatographed on paper. In another experiment, crystalline thyroxine was added to a plasma-protein butanol extract and this solution subjected to chromatography on a column. After measurement of radioactivity of the effluent fractions, photometric ninhydrin determinations were performed on each fraction (29).

RESULTS

Table I indicates the data obtained in each patient relating the dose of I^{131} and the time after its administration when the blood was sampled to the total plasma radioactivity, its separation into trichloroacetic acid-soluble and insoluble fractions, and the butanol extractability of the latter. Serial studies carried out on successive samples of blood from patient No. 6 indicating the predominance of acid-soluble inorganic I^{131} during the first eight hours following isotope ingestion and its subse-

[4] A sample of this compound, prepared by Dr. Rosalind V. Pitt-Rivers, was supplied through the courtesy of Dr. Fritz Lipmann.

TABLE I

Concentration and distribution of labelled iodine in plasma after administration of I^{131}

Patient	Dose I^{131}	Time blood drawn after I^{131} administration	Plasma I^{131} μc. per cc. per mc. administered*	Total I^{131} in plasma sample fractionated†	Trichloroacetic acid-soluble		Trichloroacetic acid-insoluble		Combined butanol extracts	
					cts./sec.†	% of total plasma I^{131}	cts./sec.†	% of total plasma I^{131}	cts./sec.†	% of trichloroacetic acid-insoluble
	(*mc.*)	(*hours*)		(μc)						
1	10	48	7.95×10^{-3}	0.134	0.5	1.4	35.7	98.6	31.6	89
2	10	48	64.5×10^{-3}	1.09	5.9	2	290.1	98	268	92
				1.08	6.4	2.2	285.6	97.8	290.8	102
3	12.5	65	22.9×10^{-3}	2.05	11.1	2.0	543.9	98	522.3	96
4	10	72	19.7×10^{-3}	1.29	12.1	3.4	335.9	96.6		
5	15	192	11.9×10^{-3}	0.358	1.7	1.8	95.1	98.2		
6	14.7	4	5.1×10^{-3}	0.222	53.7	89.5	6.3	10.5		
		8	1.44×10^{-3}	0.0621	15.0	89.5	1.8	10.5		
		24	1.22×10^{-3}	0.050	1.7	12.6	11.8	87.4		
		72	3.92×10^{-3}	0.133	—	—	—	—		
		240	8.10×10^{-3}	0.249	1.5	2.2	65.8	97.8	62.8	95

* Corrected for decay to the time of ingestion of I^{131}.
† 1 count per sec. in 1.0 cc. volume = 3.70×10^{-3} microcuries.

quent great diminution concurrent with the increase, both relative and absolute, of protein-bound I^{131} are in general agreement with the observations of McConahey and his colleagues (8). After 48 hours following administration of the radioactive iodine, 97–99 per cent of the plasma I^{131} is protein-bound. The degree of completeness of the butanol extraction of the protein-bound I^{131}, as indicated in the table, approximates 95 per cent. The method of trichloroacetic acid precipitation and butanol extraction of the suspended insoluble precipitate was more efficient and complete in removing I^{131} from the aqueous phase than the method of direct plasma extraction with butanol. In two instances (patients Nos. 1 and 2), in which by the former method 89 and 97 per cent of the acid-insoluble iodine was extracted, the latter method yielded values of 70 per cent and 89 per cent of the total plasma radioiodine, figures comparable to those reported in butanol extraction of rat plasma (12, 17, 18).

The final washing of the combined butanol extracts with dilute acid was found to be essential. This washing removed virtually no radioactivity; if it was omitted, the green butanol solution assumed a gelatinous consistency which made manipulation difficult and rendered chromatograms of the redissolved, evaporated solutions unsatisfactory, R_F values of added carriers becoming markedly diminished and the bands distorted. The nature of the green color of the butanol solutions is not clear. Preliminary attempts to extract it from the radioactivity by the use of several organic solvents were unsuccessful, and on column chromatography, the rate of downward movement of the green band is very similar to that of the radioactivity. Butanol extracts of whole plasma did not exhibit the green color.

One-dimensional paper chromatography indicated that the radioactivity in the butanol extract of the protein-bound I^{131} had an R_F value identical to that of crystalline thyroxine, whether the latter was added to the active extract chromatographed or was run with an extract parallel to those without added carriers. In every case, only one area of radioactivity appeared on the paper, at the position corresponding to the location of thyroxine. No radioactivity was detectable at the bands corresponding to diiodotyrosine, monoiodotyrosine, or iodide. This is in contrast to results obtained in chromatography of rat plasma extracts in collidine (18), where appreciable amounts of iodide and small amounts of diiodotyrosine were encountered, presumably due to decomposition of thyroxine during chromatography. No iodide spot would be expected (unless decomposition during chromatography occurred), under the conditions of the present work because *1)* the trichloroacetic acid precipitate alone is extracted, and *2)* a step in the procedure involves evaporation of an acid solution to dryness. Representative chromatograms are shown in Figure 1.

In the butanol-acetic acid-water chromatographic solvent the approximate R_F values of potassium iodide, monoiodotyrosine, diiodotyrosine, thyroxine, and acetylthyroxine are, respectively, 0.16, 0.42, 0.53, 0.78, and 0.88; the actual values vary somewhat with the age of the solvent but in every case the R_F of thyroxine was equal to the R_F of the radioactivity. The iodinated amino acid carriers can be recognized not only by their distinct R_F values but also by the differences in ninhydrin color they produce on dry paper, monoiodotyrosine being reddish-purple, diiodotyrosine blue, and thyroxine bluish-gray. Ninhydrin treatment of the chromatogram run without added carriers revealed a long, faint pink streak in the center of the paper and a very faint purple at the origin; the quantity of thyroxine in the aliquot of plasma extract chromatographed was far too minute to be detectable by a colorimetric ninhydrin reaction. The paper without carriers also presented a colorless, lipid-like, hydrophobic spot free of I^{131} at $R_F = 0.95$, detectable by the formation of water droplets on streaking this region of the paper with aqueous sodium carbonate solution. The rates of movement of diiodotyrosine and thyroxine added to butanol extracts of plasma proteins were greater than those exhibited by these substances in acid butanol solution, while monoiodotyrosine and iodide were unaffected by the presence of the plasma constituents; thus, the R_F of diiodotyrosine and thyroxine added to a dried plasma butanol extract spot were found

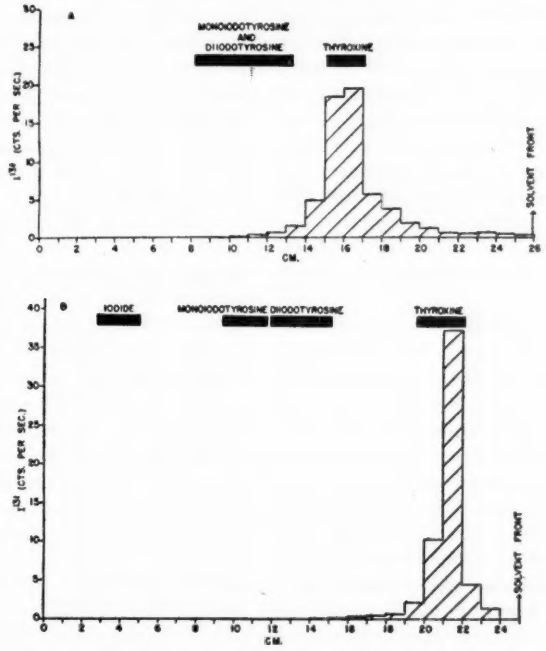

FIG. 1. DISTRIBUTION OF RADIOACTIVITY ON FILTER PAPER CHROMATOGRAMS OF BUTANOL EXTRACTS OF AQUEOUS SUSPENSIONS OF PLASMA TRICHLOROACETIC ACID PRECIPITATE

A. Solvent: *n*-pentanol : pyridine : water, 40 : 40 : 15. Carrier added.

B. Solvent: *n*-butanol : glacial acetic acid : water, 75 : 10 : 15. No added carrier.

Position of added (A) and parallel (B) carriers are indicated by shaded rectangles.

to be 0.62 and 0.83, respectively, while these crystalline components without plasma extract showed values of 0.53 and 0.78. Until the accelerating affect of plasma extract on the movement of thyroxine was appreciated, the concordance of the R_F of radioactivity with added thyroxine and the discrepancy between the R_F of activity and parallel thyroxine in solution of carrier alone was puzzling. The apparent discrepancy between results with added and parallel carriers was resolved by addition of the carrier solution designed for parallel runs to a spot of plasma extract. In the pentanol-pyridine-H_2O solvent, the R_F of thyroxine was found to be 0.61, as was that of the radioactivity; mono- and diiodotyrosine are incompletely resolved by this solvent and no radioactivity was detected in their combined area.

The R_F values obtained with butanol-acetic acid-water developing solvents were not constant, decreasing with time presumably because of esterification (30). For this reason reliance could not be placed on presumptive identification of an unknown substance by comparison with previously determined R_F values of known substances, but only with parallel carriers run at the same time and under the same conditions. It was found useful to employ solvents one or two days old, the rate of change of R_F values appearing to be slower after this time.

In addition to comparison of the area of radioactivity with thyroxine on the basis of identity of R_F, more precise localization was accomplished as follows: After the strip with added carriers had been counted and the color developed by ninhydrin, the grayish-blue thyroxine was cut out of the paper and also adjacent ninhydrin-negative paper areas of approximately equal size. The radioactivity of each piece of paper was then determined. The highest radioactivity was in the ninhydrin-colored thyroxine spot, but detectable amounts were present somewhat peripheral to the outermost zone of ninhydrin color, a reflection of the greater sensitivity of detection achieved by counting as compared with ninhydrin color development (the latter being rather insensitive when used for the demonstration of a compound of molecular weight 777 and containing only one amino group); the greater spread of radioactivity as compared with the corresponding color spot is illustrated in Figure 1.

Chromatograms prepared from direct butanol extracts of plasma were similar to those obtained with extracts of the trichloroacetic acid precipitate; only one area of radioactivity was detected on the paper, corresponding in position with thyroxine. Spraying the paper having no added carrier with ninhydrin revealed a number of discrete ninhydrin-reacting areas, all below the position of radioactivity.

Radioautographs prepared from the two-dimensional chromatograms showed complete correspondence in size, form and location of the darkened area on the film with the ninhydrin-reacted thyroxine spot on the paper, with no band visible at areas corresponding to mono- and diiodotyrosine. A faint darkening at the solvent front and a very faint rather diffuse darkening just below the front were noted.

Column chromatography of plasma extract without added carrier yielded a single, smooth effluent curve of activity which emerged in absolute position very similar to the thyroxine peak observed on passage through a similar column of butanol extract of an alkaline hydrolysate of the thyroid gland of a rat previously injected with I^{131} (27). Out of a total of 0.388 microcurie added to the column, 0.366 microcurie was included under this curve, a recovery in a single peak of 95 per cent of the total added radioactivity. Paper chromatography of the pooled fractions constituting the effluent peak demonstrated a single active spot, the R_F of which was equal to the R_F of both added and parallel crystalline thyroxine. More quantitative evidence for the identical distribution in the continuous partition system between organic and aqueous phases of the radioactively labelled molecule and synthetic thyroxine was afforded by the results of the experiment in which 1.78 mgm. thyroxine was added to the butanol extract subjected to passage through the column, and radioactivity and ninhydrin-reacting material estimated on the effluent fractions (Figure 2). The effluent radioactivity emerged in a fairly symmetrical fashion between 24 and 28 cc., with a peak at 25.4 cc., and 97 per cent of the activity added to the column was recovered in this zone; the added thyroxine emerged between the same limits, its curve paralleling that of the radioactivity with its peak at 25.4 cc., and with 106 per cent recovery as determined by its

ninhydrin equivalent. The value for each tube of the ratio $\frac{\text{thyroxine}}{I^{131}}$, experimentally determined by dividing the corrected Klett reading of ninhydrin color for each tube by the corresponding radioactive count, was found to be constant within the limits of the errors of counting and analysis, a quantitative indication of the readily apparent similarity between the curves, as illustrated in Figure 2. The slight deviation of ninhydrin points at the lower end of the descending limb of the curve in Figure 2 may be due to the presence of a small amount of endogenous ninhydrin-reacting material in the butanol extract.

DISCUSSION

The results of the experimental work indicate that virtually all the radioactive iodine in the plasma of patients with Graves' disease, who have received therapeutic doses of I^{131} two to ten days previously, exists as a single substance precipitating with the plasma proteins but easily and quantitatively separable from the precipitate, and that this substance is indistinguishable from thyroxine in its chromatographic behavior in two different partition systems. These findings are in general agreement with results obtained on the plasma iodine of rats by essentially similar methods (12, 17–19). A negative statement that would appear to be warranted is that no radioactive mono- or diiodotyrosine and no acetylthyroxine are present in plasma in significant amounts; the quantitative paper strip method as employed here is sensitive enough to detect radioactivity in the locations of these compounds if amounts as small as 0.5 per cent of the total were present, yet none was detectable either by direct counting or by radioautographs.

Chromatographic methods whether by adsorption or partition are peculiarly adapted to the unequivocal demonstration of the non-identity of an unknown compound and known substances suspected of being identical with it. Rigorous proof of the identity of an unknown substance whose be-

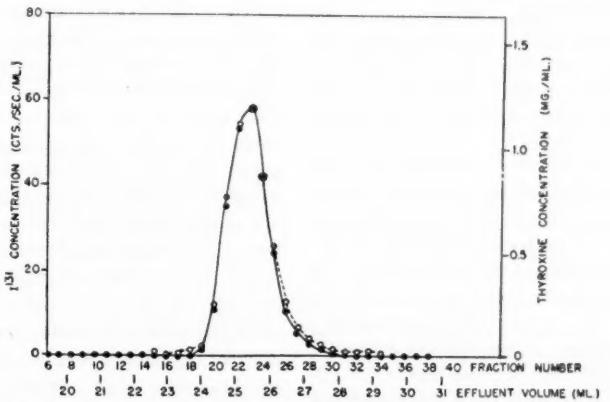

FIG. 2. EFFLUENT CURVES OF RADIOACTIVITY (SOLID CIRCLES-UNBROKEN LINE) AND OF THYROXINE (OPEN CIRCLES-BROKEN LINE) FROM A CELLULOSE COLUMN 50 CM. HIGH AND 1.2 CM. IN DIAMETER

Solvent: *n*-butanol: glacial acetic acid: water, 75: 10: 15. A concentrated butanol extract (0.35 cc.) of the trichloroacetic acid precipitate from the plasma of patient No. 3 containing 0.280 microcurie I^{131} and 1.78 mgm. added thyroxine was placed on the column. The effluent fractions between 24 and 27.5 cc. contained 0.272 microcurie and 1.89 mgm. thyroxine (ninhydrin-equivalent). For successive fractions 19 through 27, inclusive, the values of the corresponding graphical representations of the ratio

$$\frac{\text{thyroxine concentration}}{I^{131}\text{ concentration}}$$

are: 1.22, 1.07, 1.05, 1.02, 1.00, 0.99, 1.07, 1.18, and 1.29.

havior on a chromatogram seems the same as a compound of known structure on the basis of position, both absolute and in relation to other known substances, would require isolation of the unknown material, crystallization, and characterization by elementary analysis and standard chemical methods (31). The minute concentration of protein-bound iodine in plasma renders such positive methods impractical. Nevertheless, in systems in which the general nature of the compounds likely to be encountered is known, correct identification, with a high degree of probability, may be made by analysis of chromatographic behavior in comparison with known substances (32). The constancy of the relationship demonstrated between the concentration of added thyroxine and of I^{131} in the effluent fractions of a butanol extract of plasma protein is very suggestive evidence in favor of the concept that the endogenous compound is indeed thyroxine; evidence of this kind is somewhat akin to that derived from repeated crystallization to constant specific activity of labelled material with added carrier (12). The possibility, remote though it may appear, that added thyroxine and the labelled plasma constituent run *pari passu* in the column and also co-crystallize fortuitously is not excluded (12). The possibility that the paper chromatographic method may not afford a satisfactory resolution between thyroxine and a small thyroxine-peptide has been mentioned by Chaikoff and his associates (18) as a point to be investigated before unqualified acceptance can be accorded to the identity of thyroxine with the circulating thyroid hormone. In this connection the good resolution achieved in the present work between N-acetylthyroxine and thyroxine is encouraging. A considerable difference in distribution coefficients between immiscible solvents of rat plasma I^{131} and of a thyroxine-peptide of uncertain composition has been observed (12).

The probability that exchange reaction between I^{131} contained in the protein-bound iodine and added thyroxine occurs during chromatography is small, since the R_F values with and without added thyroxine are the same, and since the addition of other iodinated amino acids and iodide as carrier results in no activity in these carriers. Radiochemical exchange and decomposition have also been eliminated as confusing factors in the rat plasma (17, 19).

In order to achieve a sufficiently high concentration of plasma protein-bound I^{131} to make chromatographic study practical, administration of a dose of radioiodine much larger than the usual tracer dose is almost mandatory, and the procedure is thus necessarily limited to individuals undergoing treatment for Graves' disease. In comparable studies in animals, doses of 80–150 microcuries per rat have been employed (17, 18) which are proportionally larger than the 10–15 millicuries administered to human subjects in the present study. It is unlikely that the conclusions regarding the nature of the circulating thyroid hormone which were derived from observations made in patients receiving therapeutic doses of I^{131} would be different had the study been conducted with tracer doses potentially less destructive to the thyroid gland. Radioautographs of paper chromatograms of rat plasma extracts have revealed identical patterns whether each animal received 1 or 100 microcuries of I^{131} (20).

Whether the terms "hormonal iodine," "protein-bound iodine," and "precipitable iodine" of plasma or serum are synonymous is not completely established (33). The view that the circulating thyroid hormonal iodine comprises a large fraction rather than all of the plasma protein-bound iodine rests on the observations that there is a small but definite concentration in cases of myxedema (14, 33), that probably not all the organic iodine is ordinarily extractable with butanol and not re-extractable with Blau's reagent (13, 14), that administered organic iodine compounds such as are used in cholecystography and myelography may produce elevated protein-bound iodine concentrations for considerable periods of time (34), and that ingestion of even inorganic iodide may result in increase of the serum protein-bound iodine concentration, the increment clearly not being thyroxine as shown by its solubility properties (35). The plasma protein-bound iodine may, therefore, on occasion represent more than one component. The results of the present study of labelled plasma iodine indicate that in Graves' disease the circulating thyroid hormone is indistinguishable from thyroxine, and there is little reason to suspect a qualitative difference in the nature of the hormone in other individuals. It would therefore appear that the plasma thyroxine is more specifically

identifiable with the circulating thyroid hormone in man than is the protein-bound iodine.

SUMMARY

1. Studies of the plasma of six hyperthyroid patients two to ten days following the administration of therapeutic doses of radioactive iodine (I^{131}) indicate that 97 to 99 per cent of the labelled iodine is protein-bound.

2. Methods are described for the quantitative extraction from plasma of the labelled protein-bound iodine and for its characterization by chromatographic technics using filter paper and cellulose columns.

3. Practically all of the labelled organic iodine in the plasma has been found to exist as a single substance indistinguishable from thyroxine.

4. No monoiodotyrosine, diiodotyrosine nor acetylthyroxine were detectable as constituents of the plasma protein-bound iodine.

5. The evidence would indicate that the circulating thyroid hormone in Graves' disease is thyroxine.

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THE MECHANISM OF HEMOLYSIS IN PAROXYSMAL COLD HEMOGLOBINURIA. I. THE ROLE OF COMPLEMENT AND ITS COMPONENTS IN THE DONATH–LANDSTEINER REACTION [1, 2]

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Donath and Landsteiner demonstrated that the abnormal factor responsible for hemolysis in paroxysmal cold hemoglobinuria (PCH) is in the patient's serum (1). Inactivation of the serum by heat prevented hemolysis, but the addition of fresh serum restored the activity. The hemolytic reaction *in vitro* was shown to occur in two phases: *1*) a cold phase during which antibody (hemolysin) is adsorbed on the erythrocytes; *2*) a warm phase during which the sensitized cells are lysed in the presence of complement (fresh serum).[3]

Subsequent investigators (*2a*–4) found that the addition of fresh complement, as contained in a 1–5 or a 1–10 dilution of guinea pig serum, sometimes failed to restore the hemolytic activity of PCH sera inactivated at temperatures ranging from 45°C to 56°C. It was concluded, therefore, that the hemolysins from various patients differed in heat-stability. Furthermore, the question was raised as to the need for complement during the cold phase of the reaction (*2a*–9). Some (4–8) indicated that union of hemolysin and erythrocytes took place in the cold only in the presence of complement. Others (*2a*, 3, 9) observed hemolysis when complement was added only after the mixture of inactivated serum and erythrocytes had been chilled and warmed. On the basis of his studies (*2a*) and after reviewing the findings of others, Mackenzie (3) concluded *1*) that complement was essential for the completion of the hemolytic process, *2*) that it need not be present during the cold phase but caused greater hemolysis if present throughout the reaction, and *3*) that, when present, the "midpiece" of complement was adsorbed in the cold by the erythrocyte-lysin complex. Recently Siebens, Zinkham, and Wagley (10) reported that complement is required in both the cold and warm phases of the Donath-Landsteiner reaction. Evidence of erythrocyte-antibody inter-action was obtained not only by hemolysis but also by agglutination by antiglobulin serum.

Studies of sera from two patients have provided further data on the nature and behavior of hemolysin and on the role of complement in paroxysmal cold hemoglobinuria. These data show that this hemolytic system is unique in two respects: *a*) complement is required for antibody fixation as well as for subsequent hemolysis, and *b*) only two of the four components of complement are necessary.

MATERIALS AND METHODS

Hemolysin titration

Reagents were added for the Donath-Landsteiner reaction to give a final volume of 1 ml. as follows: 0.25 ml. of serum, 0.25 ml. of guinea pig complement and 0.5 ml. of 5 per cent suspension of erythrocytes. This method utilized equal volumes of serum and complement and a larger volume of cells than recommended by Mackenzie (*2a*). It was found to be more sensitive because the intensity of color in the supernatant was easier to estimate than the volume of remaining erythrocytes. In titration, serial two-fold dilutions of the patient's serum in buffered, physiologic saline solution (*2b*), pH 7.4, and freshly reconstituted "lyovac" guinea pig serum, diluted 1–2, were added to each tube. After addition of erythrocytes (of same group, or group O), the mixture was chilled for 30 minutes in an ice bath, warmed for 30 minutes in a 37°C water bath, centrifuged, and the supernatant read for hemolysis. Certain modifications of this procedure, such as varying the complement dilutions

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[3] Complement and fresh serum are used interchangeably throughout this paper.

and washing the erythrocytes between the cold and warm phases, are indicated in the individual protocols. Cells so washed were incubated in 0.25 ml. of the appropriate complement dilution.

Agglutination by anti-human serum rabbit serum (antiglobulin serum)

Immune rabbit serum (11) was produced by the daily intravenous injection of 0.1 ml. of group O human serum. Three seven-day courses of injections were given with an interval of one week between each course. The serum was heated at 56°C for 30 minutes, and erythrocyte agglutinins were adsorbed by incubation with pooled group A, B and O red blood cells. After three adsorptions, a 1–2 dilution of the serum did not agglutinate normal cells. When tested against Rh cells sensitized with hyperimmune Rh antibodies, the agglutinating titer of the serum was 128 to 256. The serum produced a visible precipitin reaction with a 1–15,000 dilution of human serum. An initial dilution of 1–4 anti-human serum rabbit serum was used in the following tests:

Coombs tests

Antiglobulin serum was used to detect the presence of antibody on the surface of erythrocytes. Erythrocytes sensitized *in vivo* were agglutinated in the Direct Coombs test; erythrocytes sensitized *in vitro*, by chilling in PCH serum, were agglutinated in the Indirect Coombs tests.

Direct

The patient's erythrocytes were washed three times with saline solution, made up to a 2 per cent suspension, and added in a volume of 0.2 ml. to an equal volume of serial two-fold dilutions of antiglobulin serum. After incubation for one hour at 37°C, the tubes were examined for agglutination by agitation over a magnifying mirror. The titer is expressed as the reciprocal of the highest dilution of antiglobulin serum producing agglutination.

Indirect

Two different procedures were used to detect the adsorption of antibody by erythrocytes chilled in PCH serum. In the first, (a), cells were chilled in whole serum, and then tested as in the direct Coombs test. In the second, (b), cells were chilled in different dilutions of PCH serum and tested with a constant amount of antiglobulin serum.

Indirect (*a*): PCH serum, guinea pig complement and cells were added in the same proportion, but in twice the volumes used for hemolysin titration: 0.5 ml. serum; 0.5 ml. of a 1–2 dilution of complement; and 1.0 ml. of a 5 per cent erythrocyte suspension. The mixture was chilled in the ice bath for 30 minutes and centrifuged. The cells were washed three times with cold saline solution, made up to a 2 per cent suspension, and tested for agglutination after incubation at 37°C for one hour in dilutions of antiglobulin serum. In this test, the patient's serum was not diluted and the antiglobulin serum was diluted. As in the direct Coombs test, the titer is the reciprocal of the highest dilution of antiglobulin serum producing agglutination. This titer reflects only indirectly the amount of antibody adsorbed by the cells (12).

Indirect (*b*): To determine the agglutinating capacity of the patient's serum, serial two-fold dilutions of the serum were made in 0.2 ml. of saline. Two-tenths ml. of a 2 per cent suspension of erythrocytes and 0.2 ml. of a 1–2 dilution of complement were then added. After chilling for 30 minutes in ice water, the cells were washed three times with cold saline solution and resuspended in 0.2 ml. of saline. Then 0.2 ml. of a 1–4 dilution of antiglobulin serum was added to each tube. After incubation at 37°C for one hour, the test was read for agglutination. In this test, the patient's serum was diluted and the antiglobulin serum was not. The titer, comparable to the hemolysin titer, reflects the amount of antibody in the patient's serum.

Complement titration

The lyovac guinea pig serum and human sera were tested for complement activity in a final volume of 1 ml. using 0.4 ml. of 1 per cent sheep cells sensitized with four units of amboceptor. That amount of serum producing complete hemolysis after incubation at 37°C for 30 minutes was considered to contain one hemolytic unit of complement. The amount of guinea pig complement used in the various tests is expressed as dilutions of freshly reconstituted guinea pig serum. The two lots of serum used were titered periodically, and contained one hemolytic unit of complement in 0.2 ml. of a 1–30 dilution, or about 150 units per ml.

Preparation of fractions of guinea pig serum

The four components of complement have been designated (13) by the following symbols: C'1, C'2, C'3, and C'4.

Four fractions of fresh guinea pig serum, each lacking one component of complement, were prepared by methods previously described (14, 15). In brief, complement lacking either C'1 or C'2 was prepared by dialyzing serum against acetate buffer of pH 5.2 and ionic strength of 0.02 at 1°C. The supernatant was neutralized to pH 7.2 with NaOH and sufficient NaCl was added to make a final ionic strength of 0.15; this fraction lacked C'1. The precipitate was redissolved in phosphate buffer of pH 7.2 and ionic strength of 0.15; this fraction lacked C'2. Fractions lacking C'3 and C'4 were prepared by treating serum with zymosan (16) and ammonia, respectively.

Each of the above fractions was inactive when tested at a five unit level. Full complement activity was restored at a one unit level upon the appropriate combination of the fractions.

RESULTS

Differences in behavior of the sera from two patients with characteristic clinical pictures (see appendix for case summaries) focused attention on the exact role of complement in the Donath-Land-

TABLE I

Amount of complement needed for antibody fixation and hemolysis

Constant amount of antibody

Case No.	Test	Dilution of complement					
		2	4	8	16	32	64
1	Indirect Coombs (a)	++++	++++	++	+	0	0
	Hemolysin	+++	++	+	0	0	0
2	Indirect Coombs (a)	++++	++++	+++	++	++	0
	Hemolysin	+++	++	+	0	0	0

Indirect Coombs (a) and hemolysin titration—see text under Materials and Methods.
0 = No reaction.
+ to ++++ = Increasing degrees of reaction; in agglutination test, ++++ indicates disc formation.

steiner reaction. The findings are presented essentially in the order in which the study evolved. It was found that: *1*) the Donath-Landsteiner reaction requires a large amount of complement; *2*) complement must be present during both the cold and warm phases of the reaction; *3*) a reciprocal relationship exists between the complement requirements in the two phases and between the amount of complement and the degree of antibody fixation; and *4*) the hemolytic reaction requires only two components of complement.

The need for large amounts of complement

Serum from the first patient was initially active without the addition of guinea pig complement. After heating at 56°C for 30 minutes, the hemolytic activity of the serum was not restored by the addition of a 1–10 dilution of guinea pig serum. Following prolonged storage at − 20°C, the patient's serum showed barely detectable complement activity. After the addition of a 1–10 dilution of guinea pig serum to restore complement, only slight hemolytic activity was demonstrable. It was assumed, therefore, that the hemolysin had been destroyed by heat and denatured by storage in the frozen state.

With the second patient's serum, however, a positive Donath-Landsteiner reaction could not be demonstrated even after the addition of a 1–5 dilution of guinea pig serum equivalent to eight hemolytic units of complement. The patient's serum was not anticomplementary; instead it contained one unit of complement in 0.15 ml. of a 1–10 dilution. Finally, the addition of a 1–2 dilution of guinea pig serum, equivalent to 20 units of hemolytic complement, did result in hemolysis. It was then noted that serum from the first patient could be restored to its original hemolytic activity by the addition of a 1–2 dilution of complement. In the presence of this amount of complement, the hemolysins from both patients showed maximal activity even after heating at 62°C for 30 minutes.

Such stability of the antibody permitted heat-inactivation of complement in the patients' sera, and thus the amount of guinea pig complement required for sensitization and hemolysis could be

TABLE II

Antibody levels in two patients with paroxysmal cold hemoglobinuria

Constant amount of complement

Case No.	Test	Dilution of PCH serum							
		Undiluted	2	4	8	16	32	64	128
1	Indirect Coombs (b)	++++	+++	++	++	++	++	+	0
	Hemolysin	+++	+++	+++	+++	+++	++	+	0
2	Indirect Coombs (b)	++++	+++	++	+	0	0	0	0
	Hemolysin	+++	++	+	0	0	0	0	0

Case No.	Test	Dilution of antiglobulin serum						
		4	8	16	32	64	128	256
1	Indirect Coombs (a)	++++	++++	++++	+++	+++	+	0
2		++++	++++	++++	+++	++	+	0

Indirect Coombs test (b)—see text under Materials and Methods.
See footnotes, Table I.

determined. It is apparent (Table I) that a large amount of guinea pig complement was required. Two to four times as much complement was necessary for hemolysis as was needed for antibody fixation.

There was no significant difference in the amounts of complement required to activate the two sera (Table I), although serum from case 1 had a much higher antibody titer (Table II). Thus, the amount of complement, rather than the hemolysin titer, was the limiting factor in the hemolytic reaction. Adsorption of the sera showed that agglutination of the sensitized cells by dilutions of antiglobulin serum (indirect Coombs [a]) did not distinguish between antibody levels (Table II). The erythrocytes were sufficiently sensitized in the whole sera so that the titers merely reflected the potency of the antiglobulin serum. Adsorption of antibody from dilutions of PCH serum and the use of a constant amount of antiglobulin serum for agglutination (indirect Coombs [b]), however, did reflect the hemolysin titer (Table II).

The need for complement during the two phases of the Donath-Landsteiner reaction

Studies on sera from both patients confirmed the need for complement during both the cold and warm phases of the reaction. A sample protocol is outlined in Table III. When erythrocytes were chilled in heat-inactivated PCH serum in the presence of complement, as in tubes 1 and 2, fixation of hemolysin occurred as indicated by their agglutination in a 1–128 dilution of antiglobulin serum. Cells chilled in serum alone (tube 3), or in saline (tube 4), were not agglutinated by a 1–4 dilution of antiglobulin serum.

When the sensitized cells were washed and incubated at 37°C in the presence of complement, hemolysis occurred. There was no hemolysis of sensitized cells when complement was absent during the warm phase. As shown in tube 3, the addition of complement in the warm phase did not lead to hemolysis when complement was absent during the cold phase, nor did hemolysis occur in the complete absence of complement (tube 4).

TABLE III

The need for complement during the two phases of the Donath-Landsteiner reaction

Phase	Materials	Tube No. 1	2	3	4
Cold	Erythrocytes, 5% suspension	0.5	0.5	0.5	0.5
	Inactivated PCH serum	0.25	0.25	0.25	0.25
	Complement, 1–2 guinea pig serum	0.25	0.25	0.0	0.0
	Saline solution	0.0	0.0	0.25	0.25
Agglutination in antiglobulin serum		++++	++++	0	0
Warm	Washed Rbc, 5% suspension	0.5	0.5	0.5	0.5
	Complement, 1–2 guinea pig serum	0.25	0.0	0.25	0.0
	Saline solution	0.0	0.25	0.0	0.25
Hemolysis		+++	0	0	0

See footnotes, Table I.

TABLE IV

Relationship between complement requirements in two phases of the Donath-Landsteiner reaction

Constant amount of antibody; read for hemolysis

	Dilution of complement	Warm phase 2	4	8	16	32	64	128	256
Cold phase	2	++++	++++	++++	++	+	+	0	0
	4	++++	++++	++++	++	+	0	0	0
	8	++++	++	++	+	0	0	0	0
	16	+++	++	++	0	0	0	0	0
	32	++	+	+	0	0	0	0	0
	64	++	+	0	0	0	0	0	0
	128	+	0	0	0	0	0	0	0
	256	0	0	0	0	0	0	0	0

See footnotes, Table I.

Relationship between complement requirements in two phases of Donath-Landsteiner reaction

It was noted that much less complement was needed in the warm phase when a 1–2 dilution of complement had been used in the cold phase. Erythrocytes were chilled in a constant amount of PCH serum with various dilutions of complement, washed three times with saline, and then warmed in various dilutions of complement. A reciprocal relationship existed between the amounts of complement required in the cold and warm phases (Table IV). The more complement present in the cold phase, the less was needed during the warm phase. The less complement present during the cold phase, the more was needed during the warm phase. For example, when a 1–2 dilution of complement was used in the cold phase,

hemolysis still occurred when a 1–64 dilution was used in the warm phase. When a 1–64 dilution was used in the cold phase, a 1–4 dilution was required in the warm phase.

Relationship between amount of complement and degree of antibody fixation

Complement was not only necessary in the cold phase for the fixation of antibody, but it was found that a reciprocal relationship existed between the amount of complement present and the amount of antibody fixed. This was demonstrated in two ways: *1*) by using antiglobulin serum to measure the amount of antibody adsorbed (indirect Coombs [a]), and *2*) by determining the hemolysin titer of heat-inactivated serum while varying the dilution of added complement.

TABLE V

Relationship between amount of complement and antibody fixation

Constant amount of antibody; read for agglutination

	Dilution	Antiglobulin serum						
		4	8	16	32	64	128	256
Complement	2	++++	++++	++++	+++	++	+	0
	4	++++	++++	++++	+++	+++	+	0
	8	++++	++++	++++	+++	+++	++	0
	16	+++	+++	++	++	++	0	0
	32	+++	+++	++	+	0	0	0
	64	++	++	+	0	0	0	0
	128	+	+	0	0	0	0	0
	256	0	0	0	0	0	0	0

See footnotes, Table I.

The results show that the more complement present in the cold phase, the higher the titer demonstrable on agglutination of sensitized cells by antiglobulin serum (Table V). When a 1–2 dilution of complement was used in the cold phase, the erythrocytes were agglutinated by a 1–128 dilution of antiglobulin serum. However, a 1–8 dilution of antiglobulin serum was required to agglutinate cells chilled in the presence of a 1–128 dilution of complement. When less than this amount of complement was used, no fixation of antibody was detected.

As is also true of the well-known sheep cell-amboceptor hemolytic system, the more complement present the higher the antibody titer demonstrable by hemolysis of sensitized cells (Table VI). When undiluted complement was used, the PCH serum had a hemolysin titer of 32. When 1–8 complement was employed, the hemolysin titer was only 4 and the degree of hemolysis was reduced.

TABLE VI

Relationship between amount of complement and antibody fixation

Decreasing amounts of antibody; read for hemolysis

	Dilution	PCH serum (antibody)						
		Undiluted	2	4	8	16	32	64
Complement	Undiluted	+++	+++	+++	+++	+++	+	0
	2	+++	+++	+++	+++	+	0	0
	4	+++	+++	+++	+	0	0	0
	8	+	+	+	0	0	0	0
	16	0	0	0	0	0	0	0

See footnotes, Table I.

The role of the components of complement

The fact that complement was required for union of antigen and antibody as well as for hemolysis, and that antibody fixation occurred only in the cold, marked this as an unusual antigen-antibody system. Accordingly, the behavior of the various components of complement was investigated to determine if their mode of action differed from that found in the conventional sheep cell-amboceptor hemolytic system (17).

Heat-inactivated PCH serum and erythrocytes, in the same amounts used for hemolysin titration, were chilled with the four fractions of guinea pig serum, each fraction lacking one of the components of complement (Table VII). The cells were then washed and warmed in the presence of intact complement. Cells chilled in the fractions lacking C′1, C′2, and C′3 were hemolyzed. Only cells chilled

TABLE VII

Action of guinea pig serum fractions each lacking one component of complement

Cold phase	Warm phase	Hemolysis
Missing component C′1	Intact complement	+++
C′2	Intact complement	+++
C′3	Intact complement	+++
C′4	Intact complement	0
	Missing component	
Intact complement	C′1	+++
Intact complement	C′2	0
Intact complement	C′3	+++
Intact complement	C′4	++
Intact complement	Intact complement	+++

See footnotes, Table I.

in that fraction lacking C'4 did not hemolyze. The procedure was then reversed. Inactivated PCH serum and cells were chilled in the presence of intact complement (Table VII). The cells were then washed and warmed in the four fractions of guinea pig serum. Cells warmed in the fractions lacking C'1, C'3 and C'4 were hemolyzed. Only cells warmed in that fraction lacking C'2 did not hemolyze.

When the four fractions were used in both the cold and warm phases (Table VIII), the various combinations again indicated that intact complement was not necessary for hemolysis. Hemolysis occurred if C'1 and C'3 were absent in both phases. When C'4 was missing in the cold phase, no hemolysis occurred. The weak hemolysis obtained with the fraction lacking C'3 can be attributed to the fact that this fraction contains less C'4 than the two other fractions. When C'2 was missing in the warm phase, no hemolysis occurred. There was a single exception: when C'2 was missing in the cold phase and C'4 was missing in the warm phase, no hemolysis occurred.

Since C'4 retains its activity after heating at 56°C for 30 minutes (15) it was thought that heated guinea pig serum might provide the C'4 activity needed in the cold phase of the Donath-Landsteiner reaction. This was not the case, because adsorption of antibody could not be demonstrated either by agglutination of the erythrocytes or by their subsequent hemolysis on the addition of complement in the warm phase. There was no indication that C'4 from heated serum was fixed along with "inactive" C'2, thus blocking the later action of C'2 in the warm phase. Erythrocytes, previously chilled in heat-inactivated guinea pig serum and PCH serum and washed, still adsorbed antibody and were hemolyzed in the presence of fresh complement and PCH serum.

TABLE VIII

Action of guinea pig serum fractions each lacking one component of complement

Constant amount of antibody; read for hemolysis

	Missing component	Warm phase			
		C'1	C'2	C'3	C'4
Cold phase	C'1	++	0	++	+++
	C'2	++	0	++	0
	C'3	+	0	+	+
	C'4	0	0	0	0

See footnotes, Table I.

TABLE IX

Action of human and guinea pig sera in Donath-Landsteiner reaction

Constant amount of antibody; read for hemolysis

Complement source	Dilution of serum					
	Undiluted	2	4	8	16	32
Human 1	++	+	0	0	0	0
Human 2	++	+	0	0	0	0
Human 3	++	+	0	0	0	0
Guinea pig	+++	+++	++	+	0	0

See footnotes, Table I.

Comparison of human and guinea pig complements

Since human complement obviously is sufficient for *in vivo* hemolysis and may be adequate for *in vitro* hemolysis, comparison was made of the activity of intact human and guinea pig complements. Three undiluted human sera of the same blood group as the PCH serum were used as complement in the Donath-Landsteiner reaction. All undiluted sera produced hemolysis, but when the sera were diluted 1–2, hemolysis was weak or absent. All sera, however, possessed a high titer of complement activity for a sheep erythrocyte-amboceptor system; each contained one unit in 0.15 ml. of a 1–15 dilution. Guinea pig complement titrated at the same time showed one unit in 0.15 ml. of a 1–25 dilution, *i.e.*, the guinea pig complement was nearly twice as active as the human complement.

The three human sera and the guinea pig serum were then used in varying dilutions as the source of complement in the Donath-Landsteiner reaction (Table IX). The guinea pig serum was four times as active as the human sera. Since the C'2 and C'4 components are essential for the Donath-Landsteiner reaction, these findings are in keeping with the fact that the differences in activity between guinea pig and human sera may be due to the lower content of C'2 and C'4 in human serum (18).

DISCUSSION

The need for complement in the hemolytic reaction of paroxysmal cold hemoglobinuria was recognized by Donath and Landsteiner (1) when

they first described their studies on three cases. This requirement has been repeatedly confirmed (2–10), and Mackenzie (3) reported that some of the failures to obtain positive Donath-Landsteiner reactions in patients having otherwise typical manifestations of the disease were due to the absence of complement in the patient's serum.

The experiences with the serum of case 2 were somewhat similar. This serum contained enough complement for *in vivo* hemolysis and showed complement activity when tested with a sheep-cell amboceptor system. Nevertheless, the addition of the conventional 1–10 or 1–5 dilution of guinea pig serum would not produce a positive Donath-Landsteiner reaction. It then became apparent that unusually large amounts of complement may be necessary for *in vitro* hemolysis. The use of an adequate amount of complement also restored the activity of serum (case 1) in which the hemolysin had been previously thought to have been inactivated by heat. Thus, the use of an inadequate amount of complement not only failed to detect PCH hemolysin in the serum from one patient, but also led to the erroneous conclusion that the hemolysin in the serum from another patient was heat-labile. When adequate amounts of complement were used, the hemolysins from both patients were found to be stable at 62°C. It is not possible to generalize on the basis of a study of only two cases,[4] but this experience offers an explanation for the previously noted thermolability of the hemolysins from some patients. It is suggested, therefore, that the reported variations in the heat-stability of the hemolysin in paroxysmal cold hemoglobinuria may be attributed to inactivation of human complement and the failure to add adequate amounts of the components necessary for the activity of the hemolysin.

The results indicate further that full restoration of complement activity is necessary for measurement of the hemolysin titer. Quantitation of the hemolysin requires an excess of complement, for a reciprocal relationship exists between the amount of complement present and the antibody titer.

In the presence of an adequate amount of complement, the antibody titer can be measured in two ways. Hemolysin titration in the Donath-Landsteiner reaction provides one criterion of antibody level. Agglutination by antiglobulin serum of cells sensitized in dilutions of PCH serum (indirect Coombs [b]) provides a second criterion. The results obtained with the two methods are comparable, although the agglutination titer is occasionally one dilution higher than the hemolysin titer. Since an excess of complement is used for both hemolysin and indirect Coombs titrations, this difference can not be attributed to the fact that less complement is needed for antibody fixation than for subsequent hemolysis. The slightly higher titers obtained with the indirect Coombs test (b) probably mean that less antibody is needed on the surface of the erythrocyte to react with antiglobulin serum than is needed to induce hemolysis.

It is clear that adsorbing a whole serum and then testing the erythrocytes in dilutions of antiglobulin serum (indirect Coombs [a]) does not accurately measure the antibody content of that serum. In certain cases of acquired hemolytic anemia direct testing for erythrocyte antibody may be the only method available (12, 19–22). When serum antibody is present erythrocytes should be sensitized in serial dilutions of the serum and then tested with potent antiglobulin serum (23).

In agreement with other reports (4–8, 10) it was noted that complement is essential during both the cold and warm phases of the Donath-Landsteiner reaction. Furthermore, there is a reciprocal relationship between the amounts of intact complement required in these two phases. It is possible that failure to recognize this relationship accounted for some of the discrepancies relative to the need for complement in the cold phase.

It has long been known that hemolysis is dependent on complement in the usual erythrocyte-amboceptor system. In the absence of complement, antigen and antibody will still combine as evidenced by agglutination. Complement may accelerate other antigen-antibody systems (24), but the reactions occur in the absence of complement. In contrast, the immunological character of paroxysmal cold hemoglobinuria is unique in that

[4] Since the preparation of this report, the heat stability of the antibodies in the sera from four other cases has been tested. All were found to be active after heating at 56°C for 30 minutes. The hemolysin in the serum of one patient (case 1 of Siebens, Zinkham, and Wagley [10]) was initially reported to be more thermolabile than complement when heated at 56°C.

it requires complement for antibody fixation as well as for subsequent hemolysis. Characterization of the PCH antibody has not helped to explain this phenomenon, since the hemolysin is a gammaglobulin of the pseudoglobulin type (25) and thus falls in the same class as other antibodies.

The PCH hemolytic system is unique in another respect because all components of complement are not necessary for hemolysis. In conventional hemolytic antigen-antibody reactions, the four components are all required for hemolysis and act as follows (17): C'4 combines with the red cell first or simultaneously with C'1, and the fixation of C'4 predetermines the hemolytic activity of combining C'1. The fixation of C'4 is accompanied by the fixation of C'2. The erythrocyte-antibody complex is thus rendered amenable to the action of C'3 and hemolysis results. In the erythrocyte-PCH hemolysin system, however, C'1 and C'3 are not essential. C'4 apparently combines in the cold phase, and hemolysis occurs when C'2 is present in the warm phase. These findings may appear to be at variance with Mackenzie's statement that it is chiefly, if not exclusively, "midpiece" of complement which fixes in the cold (3). This conclusion was based on the use of the sheep cell-amboceptor reaction as an indicator system for measuring residual complementary activity. "Midpiece" as formerly prepared, contains C'1, C'3 and C'4. Since the absence of any one of these components in Mackenzie's indicator system would have prevented hemolysis, Mackenzie may have been measuring the fixation of C'4, which would be in accord with the present findings.

Since only two components of complement are required for hemolysis in paroxysmal cold hemoglobinuria, titration of complement in a patient's serum by the sheep cell-amboceptor system may not measure the capacity of that serum to produce hemolysis with the PCH antibody. Although such a titration would detect the presence of all the components, it would not distinguish one absent component from another. One could then erroneously conclude that spontaneous attacks of hemoglobinuria do not require complement (26).

Another problem relative to the action of complement is evident from the results obtained with serum from case 2. This patient had spontaneous attacks of hemoglobinuria, indicating that his serum contained adequate complement for *in vivo* hemolysis. A positive Donath-Landsteiner reaction could not be obtained with the patient's serum alone, however, indicating that his serum did not contain enough complement for *in vitro* hemolysis. Yet his serum showed a high level of complement activity against sensitized sheep cells. The most obvious explanations for these findings are first, that less complement is required for PCH hemolysis *in vivo* than *in vitro*, and second, that the sheep cell-amboceptor system does not quantitate the components of complement needed for *in vitro* hemolysis in the Donath-Landsteiner reaction. Human serum does promote hemolysis in this reaction, but it is less active than guinea pig serum. This finding is in keeping with the observation (18) that guinea pig serum contains more C'2 and C'4 than does human serum.

C'2 and C'4 are now thought to be closely related, existing as a carbohydrate-protein complex in a muco-euglobin isolated from guinea pig serum (14–17). It is probable that the function of these two components in paroxysmal cold hemoglobinuria is more complex than the present studies indicate. This is suggested by the observations that when C'2 is missing in the cold phase and C'4 is missing in the warm phase, no hemolysis occurs, and that heated guinea pig serum fails to supply C'4 in the cold phase. It is obviously more desirable to measure the activity of a component by its presence than by its absence. Complete elucidation of the mechanism will be sought by obtaining purified antibody and single, pure components of complement. Such studies should clarify the reciprocal relationship between the need for complement in the two phases of the Donath-Landsteiner reaction. More than one component of complement may be fixed when intact complement is used in the cold phase, and there may be no reciprocal relationship between single components of complement. The availability of single components will also permit evaluation of their effect on the intensity of the hemolytic reaction. Purified C'4 will be necessary to understand the failure of heated guinea pig serum to supply C'4 in the cold phase.

The Donath-Landsteiner reaction is apparently dependent on a more unusual type of hemolytic mechanism than was originally thought to be the case. Not only is the antibody produced to homologous tissue components in an unexplained

manner, but the complement requirements are unique among the known antigen-antibody hemolytic systems. It is obvious that the present report has only clarified some of the qualitative aspects of the kinetics of PCH hemolysis. It is hoped that the availability of purified PCH antibody and single components of complement will permit elucidation of the PCH hemolytic system.

SUMMARY

Studies of sera from two patients with paroxysmal cold hemoglobinuria (PCH) have shown that large amounts of complement may be necessary for hemolysis in the Donath-Landsteiner reaction. By using an adequate amount of complement, the hemolysins from both patients were found to be stable at 62°C.

An excess of complement is necessary for antibody titration, since a reciprocal relationship exists between the amount of complement present and the antibody titer. Agglutination by antiglobulin serum of erythrocytes sensitized in dilutions of PCH serum provides another measure of antibody level, the titers obtained being comparable to those found by hemolysin titration in the Donath-Landsteiner reaction.

Complement is essential in both the cold and warm phases of this reaction, and there is a reciprocal relationship between the amounts of intact complement required in these two phases. The erythrocyte-PCH antibody system is unique in that it requires complement for antibody fixation as well as for subsequent hemolysis.

The PCH hemolytic system is unusual in another respect, because all components of complement are not necessary for hemolysis. Hemolysis occurs in the absence of C'1 and C'3. Hemolysis does not occur when C'4 is missing in the cold phase, or when C'2 is missing in the warm phase.

Since only two components of complement are required for hemolysis in PCH, titration of complement in a serum by the sheep cell-amboceptor system, which requires all four components, may not measure the capacity of that serum to produce hemolysis with PCH antibody.

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APPENDIX

Case 1. The patient, a 30 year old white waitress, entered the hospital in April, 1948. During the four preceding winter months she had experienced eight to ten attacks characterized by chills, nausea and vomiting, pain in the back and the passage of red urine. Each attack was precipitated by exposure to cold in addition to exercise, usually after a walk to and a long wait at a bus stop. She had been married to a sailor for five years. The husband gave no history of venereal disease but was found to have a positive serological test for syphilis.

Physical examination revealed that the temperature was 37.5°C, the pulse 84, respirations 20, and blood pressure 105/65. The right pupil was smaller than the left, and both reacted sluggishly to light. There were no other significant abnormalities.

The laboratory findings were as follows:

Urine: no cells or albumin; benzedine and porphyrin tests negative; urobilinogen, 0.64 Ehrlich units in two-hour specimen; urea clearance, 75 per cent in two hours. Blood: erythrocytes, 3,200,000; hemoglobin, 12 gm.; leucocytes, 8,700; differential count: neutrophiles, 60 per cent; lymphocytes, 33 per cent; monocytes, 5 per cent; eosinophiles, 2 per cent. Her erythrocytes were group O, Rh positive. Erythrocyte osmotic fragility, bleeding time and clotting time were normal. Ham's acid hemolysis test was negative. Wassermann and Kline tests were positive. The Donath-Landsteiner reaction was positive, the hemolysin titer being 64. The cold hemagglutinin (*27*) titer was 32. Spinal fluid: cell count, 130, of which all were mononuclear cells; protein 100 mg./100 ml.; gum mastic 443210000; Wassermann, positive. A roentgenogram of the chest was normal.

The patient was afebrile and asymptomatic during the first week in the hospital. Daily urinalyses were normal. Her hand was placed in ice water for 20 minutes. The hand became swollen, but no other change was detected. The application of cold, wet sheets to her body likewise failed to induce an attack. She was then placed in a cold room at 5–6°C for 25 minutes. During this time she exercised for two five-minute periods by stepping up and down on a stool. At the end of the chilling period, the patient stated that she did not feel cold. The vessels of her sclerae, as observed with a slit lamp, initially showed constriction; as the vessels became dilated, the blood flow was sluggish and aggregation of erythrocytes was apparent. She was put to bed and covered with blankets. Fifty minutes after leaving the cold room, she had a shaking chill and her temperature rose to 38.5°C. The period of rigor lasted for five minutes; a feeling of chilliness persisted for another 30 minutes; she had no nausea, vomiting, or backache. There was no change in the color of the urine; a specimen voided 15 minutes after the beginning of the reaction gave a negative benzedine test; a specimen voided 40 minutes later was faintly positive. Serum hemoglobin levels rose as follows: before chilling, 6 mg./100 ml.; ten minutes after chilling, 62 mg./100 ml.; two hours after chilling, 56 mg./100 ml.

Blood hemoglobin fell from 12.1 gm. to 11.1 gm.; the hematocrit fell from 39 per cent to 36 per cent. No significant change in urine urobilinogen was detected. She stated that the attack was not as severe as those that occurred prior to hospitalization. As soon as the feeling of chilliness disappeared, she felt well, although she remained febrile for three hours.

She was treated with 2 ml. of bismuth subsalicylate, intramuscularly, twice weekly for two weeks and then given a total of 9.0 million units of penicillin. Lumbar puncture showed a drop of the mononuclear cell count from 130 to 28. Exposure to cold with exercise was repeated in the cold room, but the patient had no detectable reaction. The hemolysin titer was unchanged. She was symptom free during the month (May) following discharge at which time she left the city and further observation was not possible.

Case 2. The patient, a 27 year old colored male, entered the hospital in November, 1949, because he had voided red urine on two occasions. These attacks occurred a week apart and were both associated with exposure to cold while working outdoors. His job as a salvage inspector kept him indoors, and he believed that his exposures to cold on two successive Saturday afternoons were the first of the winter. After working in the cold for about an hour, he noted frequency and urgency and observed that his urine was pink. After working longer, he remained indoors. Shortly after he began to feel warm, he had a shaking chill, his skin "prickled," "tingled" and was sensitive to touch, and he voided dark red urine. He had no rash, backache, nausea or vomiting. Successive urine specimens showed less and less color, and he soon felt well. He had a penile lesion one year before and was told that microscopic and serological tests were negative.

Physical examination revealed no significant abnormalities. The temperature was 37°C, the pulse 72, respirations 18 and blood pressure 120/70.

The laboratory findings were as follows:

Urine: normal. Blood: erythrocytes, 5,287, 000; hemoglobin, 15.3 gm.; leucocytes, 5,500; differential count: neutrophiles, 72 per cent; lymphocytes, 21 per cent; monocytes, 7 per cent. His erythrocytes were group B, Rh positive. Erythrocyte osmotic fragility was normal. Ham's acid hemolysis test was negative. Wassermann and Kline tests were positive. The Donath-Landsteiner reaction was positive, the hemolysin titer being 4. The patient's washed erythrocytes were agglutinated by antiglobin serum (positive, direct Coombs test). Cold hemagglutinins (27) were not detected in a dilution of 1–4. The spinal fluid was normal. A roentgenogram of the chest and intravenous pyelograms were normal.

Unless chilled, the patient remained afebrile and asymptomatic. Hemoglobinemia and hemoglobinuria were easily produced by placing his hand in ice water (positive Rosenthal test). This was done on three occasions, and each time there was also a leucopenia and fall in hematocrit. Twice the patient had a shaking chill and noted the skin sensation previously described. His blood pressure rose from 120/64 to 130/84. There was no increase in temperature. Except for discomfort in the chilled hand, the patient felt well within an hour. Hemoglobinuria persisted for about three hours.

The patient was treated with 7.2 million units of penicillin. A total of 1,500 ml. of blood have subsequently been removed, 1,000 ml. being replaced by an equal amount of whole blood of the same type. There has been a slight fall in the quantitative Kline titer, but no detectable change in the hemolysin titer. He has continued to have attacks of hemoglobinuria on exposure to cold.

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THE MECHANISM OF HEMOLYSIS IN PAROXYSMAL COLD HEMOGLOBINURIA. II. OBSERVATIONS ON THE BEHAVIOR AND NATURE OF THE ANTIBODY[1]

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The abnormal factor responsible for hemolysis in paroxysmal cold hemoglobinuria (PCH) is present in the patient's serum (1). As a result of infection with the spirochete of syphilis (2, 3), the patient produces a factor that, under proper conditions, hemolyzes his own erythrocytes. Hemoglobinuria is thus a dramatic manifestation of the ability of an infectious agent to induce the body to destroy one of its tissues. Such "auto-immunization" is not dependent on any specific blood group antigen (2, 3), and the serum antibody is active not only against the patient's erythrocytes but also against erythrocytes from other individuals.

The hemolysin is activated in an antigen-antibody-complement system (1–4). When erythrocytes and PCH serum are chilled, antibody fixation occurs in the presence of complement. When the sensitized erythrocytes are warmed in the presence of complement, hemolysis occurs. Recent studies have indicated that this system is not only unique in requiring complement for antibody fixation as well as for subsequent hemolysis, but that it is also unusual in requiring only two of the four components of complement for hemolysis. Hemolysis occurs in the absence of two components, C'1 and C'3. Hemolysis does not occur in the absence of components C'2 and C'4. C'4 is apparently required in the cold phase, C'2 in the warm phase (4).

It was also found that the hemolysins from two patients were stable at 62°C, apparent heat-lability being attributable to failure to use sufficient complement for reactivation (4). The use of antiglobulin serum (4–6) provided a new technique for identifying and measuring this abnormal antibody. The present report concerns further investigations on the behavior of the antibody, in the course of which it seemed desirable to attempt the isolation and characterization of the hemolysin.

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MATERIALS AND METHODS

The case histories of the two patients with paroxysmal cold hemoglobinuria have been summarized elsewhere (4). One of the patients, case 1, was observed before antiglobulin serum was available, and her erythrocytes were not tested in the direct Coombs test. Specimens of her serum, stored for approximately ten months at −20°C, were available. Thus, erythrocytes from case 2 and sera from both cases were tested.

The methods used for the following procedures were those previously described (4): *1*) Hemolysin titration, *2*) Direct Coombs test, *3*) Indirect Coombs tests, (a) and (b), and *4*) Complement titration. Plasma fractions were assayed for antibody by substituting each of the fractions for PCH serum in these tests.

Fractionation of Plasma

In a series of orientation experiments, 400 ml. of plasma from case 2 were dialyzed against repeated changes of acetate buffer of pH 5.3, ionic strength 0.02, at 0°C. After dialysis for 72 hours the precipitate was removed by centrifugation at 0°C, washed two times with pH 5.3 buffer and redissolved to a volume of 80 ml. in M/15 phosphate buffer of pH 7.4 (Fraction PI).

Aliquots of the material soluble at pH 5.3 (SI) were dialyzed against buffers between pH 5.0 and 7.0, ionic strength 0.02, for 72 hours at 0°C, and the precipitates removed by centrifugation. The remainder of SI was fractionated with certain modifications according to the methods developed by Cohn and his associates (7–9) into Fractions II + III, IV, and V. Methanol was employed instead of ethanol, however, as the organic precipitating agent. Four hundred and fifty ml. of SI were adjusted to ionic strength 0.1 with sodium chloride and to pH 6.9 with sodium hydroxide. The material was chilled to the freezing point and methanol, previously chilled to −25°C, was added slowly with stirring. The temperature was maintained near the freezing point, and at −5°C at the end of the alcohol addition. The mixture was allowed to stand overnight in a −5°C bath and then

centrifuged at −5°C for one hour at 4000 r.p.m. The precipitate (II + III) was dissolved up to 80 ml. in 0.15 sodium acetate. Sufficient thrombin was added, and the fibrin was removed by centrifugation. The material soluble at pH 6.9, ionic strength 0.27 in 25 per cent methanol (SII + III) was adjusted to pH 5.7 with acetic acid, and sufficient alcohol was added to bring the final concentration to 40 per cent. The precipitate (IV) was removed by centrifugation at −5°C for one hour at 4000 r.p.m. and dissolved to a final volume of 80 ml. in 0.15 sodium acetate. The material soluble at pH 5.7 in 40 per cent methanol (SIV) was adjusted to pH 4.7 with acetic acid. Sufficient alcohol was again added to maintain the concentration at 40 per cent. The material insoluble (V) under these conditions was removed by centrifugation, and dissolved up to 160 ml. in sodium acetate.

Sixty-seven ml. of II + III were dialyzed against repeated changes of water at 0° for 96 hours. The precipitate (II + IIIE) was removed by centrifugation at 0°C for one hour at 4000 r.p.m. and dissolved up to 67 ml. in phosphate buffer of pH 7.4. The material soluble in water was labeled II + IIIS. Because PCH antibody was distributed between II + IIIS and II + IIIE, these fractions were combined (II + III) and were fractionated according to the methods of Deutsch and his associates (10) with certain modifications. One hundred ml. of II + III were adjusted to pH 5.1 with acetic acid and sufficient water added to bring the final volume to 700 ml. at 0°C. Methanol was added to bring the final alcohol concentration to 17 per cent. The final conditions were pH 5.1, ionic strength 0.01, methanol concentration 17 per cent at −5°C. The material insoluble (III − I) under these conditions was removed by centrifugation at −5°C, and dissolved up to 25 ml. in phosphate buffer at pH 7.4. The material soluble under the above mentioned conditions was precipitated at pH 7.0, 25 per cent methanol, ionic strength 0.01 at −5°C. This precipitate was removed by centrifugation and labeled II. Fractions III − I and II were dissolved in water, dried from the frozen state, and reconstituted to 25 ml. in phosphate buffer of pH 7.4. Five ml. of Fraction II were dialyzed for 72 hours against 4 liters of distilled water at 0°C. The precipitate was removed by centrifugation and labeled IIE. The supernatant was labeled IIS.

RESULTS

The antibody in the sera of patients with paroxysmal cold hemoglobinuria can be detected and measured not only because it acts as a hemolysin but also because its presence on erythrocytes renders the cells agglutinable in antiglobulin serum (4, 6). The patient's red cells and control cells adsorb antibody *in vitro* when chilled with complement in PCH serum, and such sensitized cells are then hemolyzed when warmed with complement (Donath-Landsteiner reaction) or agglutinated when incubated with antiglobulin serum (indirect Coombs test).

It was noted that erythrocytes from case 2 were directly agglutinable in antiglobulin serum. Despite this indication of *in vivo* "sensitization," there was no evidence of *in vivo* hemolysis, nor were the direct Coombs-positive cells, presumably already coated with antibody, hemolyzed *in vitro* when warmed with complement. Variations in the agglutinability of the patient's cells occurred following induced attacks of hemolysis although no change in serum antibody was detected. These findings raised a number of questions: *a*) what is the nature of the abnormal factor ("cell antibody") adsorbed *in vivo*? *b*) is the "cell antibody" detected in the direct Coombs test the same as the serum antibody detected in the indirect Coombs test? *c*) is the serum antibody detected in the indirect Coombs test the same as the hemolysin? *d*) what is the nature of the serum antibody? The findings are presented by detailing the observations that prompted these questions and by describing the studies carried out in an attempt to answer them.

Variation in agglutinability of PCH erythrocytes in antiglobulin serum (direct Coombs test) following attacks of hemoglobinuria

When erythrocytes initially obtained from case 2 were washed and incubated in antiglobulin serum (direct Coombs test), the cells were strongly agglutinated. The patient's erythrocytes, drawn five days after his last attack of hemoglobinuria, and promptly separated from defibrinated blood without chilling, were agglutinated by a 1–128 dilution, the effective titer (4), of the antiglobulin serum. The Donath-Landsteiner reaction was positive, the hemolysin titer was 4, and the serum contained one unit of complement activity in 0.15 ml. of a 1–10 dilution. The Coombs test indicated that there was an abnormal serum protein, presumably the PCH hemolysin, on the surface of the erythrocytes. Although the cells were "sensitized" and complement was present, there were no signs of *in vivo* hemolysis. Hemolysis occurred, however, when the patient was chilled.

The detailed hematological changes will be described elsewhere (11). It need only be noted here that the maximum levels of serum hemoglobin were detected in the specimens obtained im-

mediately after the ten-minute periods of chilling. The output of urine hemoglobin was maximum in the first hour after chilling. Although the hemolysis of erythrocytes occurred promptly, it is apparent (Table I) that the change in the direct Coombs titer was delayed. During and two and four hours after each of three induced attacks, there was no significant change in the agglutinability of the patient's erythrocytes in antiglobulin serum. In each instance, however, the cells were no longer agglutinable six hours after the attack. After the first two attacks, the direct Coombs test again became positive after intervals of two and 14 days, respectively. The patient was discharged after the third induced attack and has continued to suffer spontaneous attacks when exposed to cold. The direct Coombs titer has never returned to its original level, but the patient's erythrocytes have continued to show varying agglutinability in antiglobulin serum (Table I).

There was no significant change in the antibody content of sera obtained before, during and after attacks, as measured by both hemolysin titration and the indirect Coombs test (b). The same was true of similar sera from case 1. At a time when the direct Coombs test was negative, the patient's serum showed complement activity (one unit in 0.2 ml. of 1–15 dilution) against sensitized sheep cells. The role of the individual components of complement (4) was not appreciated at that time, and the activity of components was not measured.

The reversion of the cells from Coombs-positive to -negative was not due to the fact that they had become refractory to the adsorption of antibody. Such cells, tested in serum drawn at the same time, behaved like normal cells in the indirect Coombs tests and the Donath-Landsteiner reaction. Nor did the patient's Coombs-negative cells adsorb any component from normal serum which made them Coombs-positive.

Comment: In certain cases of acquired hemolytic anemia the severity of hemolysis parallels the degree of erythrocyte sensitization (12). One would logically expect the PCH sensitized cells to be destroyed during the period of active hemolysis and to disappear from the circulation. In the instance described, on the contrary, sensitized cells remained in the circulation long after hemolysis had ceased, and the subsequent disappearance of evidence of sensitization could not be ascribed to red cell destruction. No concomitant change in serum antibody level was detected. Exhaustion of the complement component necessary for antibody fixation is a theoretical explanation (3, 4) yet to be tested, but it seems an unlikely possibility since there was no qualitative change in the complement activity of the patient's serum. There is, therefore, no satisfactory explanation for the disappearance of the direct agglutinability of the patient's erythrocytes.

A clue as to why the cells were directly agglutinable was obtained when it was noted that the direct Coombs test again became positive, following the first induced attack, after the patient's ward had been chilled while window-washers were at work. No such incident, however, was noted after the second induced attack. The patient continued to have spontaneous attacks following discharge, and as long as cold weather persisted, his cells were often agglutinable in the direct Coombs test. With the advent of warm weather attacks ceased and his erothrocytes were no longer Coombs-positive. These events suggest that the *in vivo* "sensitization" detected in the direct Coombs test was induced by chilling, but that, as mentioned below, additional chilling in PCH serum was necessary to cause hemolysis.

In vitro *behavior of erythrocytes "sensitized" (direct Coombs-positive)* in vivo

Hemolysis: Because erythrocytes obtained from case 2 were directly agglutinable by antiglobulin serum without chilling *in vitro* in PCH serum, it was thought that these cells might be hemolyzed in the warm phase of the Donath-Landsteiner reaction. The patient's Coombs-positive, "sensitized" cells were treated as follows in an effort to produce *in vitro* hemolysis:

a) 0.25 ml. of 5% cell suspension + 0.25 ml. of 1–2 guinea pig complement; incubated 37°C for 30 minutes.
b) 0.25 ml. of 5% cell suspension + 0.25 ml. of 1–2 guinea pig complement; chilled in ice bath for 30 minutes and incubated 37°C for 30 minutes.
c) and *d*) Above steps repeated with addition of 0.25 ml. of fresh normal human serum.

No hemolysis occurred. The patient's cells and control cells were then chilled and warmed with guinea pig complement in the patient's serum (Donath-Landsteiner reaction). The cells were

hemolyzed, and the degree of hemolysis of patient and control cells was the same.

The patient's erythrocytes, apparently already coated with antibody, and normal cells were then chilled in separate samples of the patient's serum, washed and tested with antiglobulin serum. Both patient and control cells were agglutinated by a 1–128 dilution of the antiglobulin serum. It thus was not possible by this technique to indicate that the direct Coombs-positive cells had adsorbed additional antibody.

The behavior of the patient's cells was further compared with normal erythrocytes. Cells from two other group B individuals and from three group 0 individuals showed the same capacity as the patient's cells for adsorbing antibody and were equally sensitive to hemolysis. Furthermore, in the Donath-Landsteiner and indirect Coombs tests, there was no demonstrable difference between the patient's cells whether they were or were not initially agglutinable in the direct Coombs test.

Since erythrocyte age is an important factor in normal cell destruction, the ability of the PCH hemolysin to lyze all erythrocytes was tested. When a small volume of cells was used in the Donath-Landsteiner reaction (2, 4) nearly all of the cells were lyzed. When a larger volume of cells was used (4), hemolysis was not complete. The cells remaining after the first incubation were washed and again chilled and warmed in PCH

TABLE I

Variations in agglutinability of PCH erythrocytes in antiglobulin serum following attacks of hemoglobinuria

Date	Time	Dilution of antiglobulin serum						
		4	8	16	32	64	128	256
11/9/49		++++	++++	++++	++++	++	++	0
11/10		++++	++++	+++	++	+	+	0
11/14	Before chill	++++	++++	+++	++	+	0	0
	9:07–9:25	Hands in ice water						
	9:27	++++	++++	++++	++++	++	+	0
	9:29	Beginning of reaction						
	10:00	++++	++++	++++	+++	+++	+	0
	3:15	0	0	0	0	0	0	0
11/15		0	0	0	0	0	0	0
11/16		+	+	+	0	0	0	0
11/17		++++	++++	++++	+++	++	+	0
11/18	Before chill	++++	++++	++++	+++	++	+	0
	9:25–9:35	Hands in ice water						
	9:37	++++	++++	+++	+++	++	0	0
	10:00	No systemic reaction; voided pink urine						
	11:35	++++	++++	+++	+++	++	0	0
	1:35	++++	+++	+++	+++	++	+	0
	3:35	+	+	0	0	0	0	0
11/19–11/30		Daily tests showed no agglutination						
12/2	Before chill	++++	++++	+++	+++	+++	+	0
	10:05–10:17	Hand in ice water						
	10:18	++++	+++	+++	++	++	+	0
	10:32	Beginning of reaction						
	12:22	+++	+++	+++	++	++	+	0
	4:30	+	0	0	0	0	0	0
12/11		Mild attack following discharge						
12/17		+++	+	+	0	0	0	0
1/1/50		Six (6) mild attacks, the last on this date						
1/4		+++	++	++	+	0	0	0
1/5		Mild attack						
1/7		+	0	0	0	0	0	0
		Venesection 500 ml.; severe attack following exposure to cold while returning home						
2/4		++	0	0	0	0	0	0
2/11		+	0	0	0	0	0	0
		1000 ml. replacement transfusion						
2/19		Mild attack						
3/11		+++	+++	+	0	0	0	0
3/18		Mild attack						
4/8		0	0	0	0	0	0	0
5/6		0	0	0	0	0	0	0
6/17		0	0	0	0	0	0	0

+ to ++++ = Increasing degrees of agglutination; ++++ indicates disc formation.

serum and complement. All erythrocytes were hemolyzed following the third incubation.

Elution: Because serum antibody can be eluted from erythrocytes (see below), an attempt was made to elute "cell antibody" from the patient's direct Coombs-positive cells. Erythrocytes from case 2 showing agglutinability in a 1–128 dilution of antiglobulin serum were suspended in saline, heated at 56°C for five minutes, and then washed. The cells were no longer agglutinated by a 1–4 dilution of antiglobulin serum suggesting that the antibody had been eluted, but no antibody could be demonstrated in the supernatant fluid.

Comment: The nature of the cell antibody could not be determined. While the direct Coombs test indicated only whether cell antibody was present or absent, these changes in agglutinability of the patient's cells could be correlated with clinical events. Chilling seemed to be responsible for the *in vivo* "sensitization" detected by the direct Coombs test. More severe chilling produced hemolysis, and this was followed by a delayed, but consistent, disappearance of *in vivo* "sensitization." Certain of the results obtained when the direct Coombs-positive cells were tested *in vitro* could, in turn, be related to the observations *in vivo*.

The patient's washed cells were agglutinated in high dilutions of antiglobulin serum; after the erythrocytes were chilled in PCH serum, no additional antibody on the surface of the cells could be detected with antiglobulin serum (indirect Coombs test [a]). This test does not distinguish between cells chilled in sera with an eight to 16-fold difference in antibody titer (4) and it may be that the patient's "sensitized" cells did adsorb more antibody. At least a change of antigen-antibody union had occurred, for the cells now hemolyzed when warmed with complement. It is evident that the antibody fixed *in vivo* did not block the *in vitro* action of antibody from the same serum. Nor did it block *in vivo* action of the antibody, since the patient had hemolytic crises after chilling.

An explanation for the behavior of the patient's erythrocytes can only be surmised. Although already "sensitized," the cells had to be chilled in PCH serum, both *in vitro* and *in vivo*, before hemolysis would occur. Since less complement is needed in the cold phase of the Donath-Landsteiner reaction for antibody fixation than is needed for subsequent hemolysis (4), it may be that chilling was necessary to fix more complement. When the "sensitized" cells were both chilled and warmed in complement, however, hemolysis did not occur. Other possible explanations are that more antibody had to be fixed, that reorientation of the erythrocyte-complement-antibody complex was necessary to promote hemolysis, or that a factor other than PCH antibody had been adsorbed by the cells. Because the substance responsible for direct agglutination of the patient's cells in antiglobulin serum could not be demonstrated in eluates, identification of this substance as the PCH serum antibody was not possible. Since, as indicated later, the serum factor responsible for agglutination in the indirect Coombs tests is the PCH hemolysin, the simplest working hypothesis is to assume that the substance reacting in the direct Coombs test is also PCH antibody.

Elution of antibody; effect of heat on agglutinability of erythrocytes sensitized in vitro

Blood group agglutinins (13) and Rh blocking antibodies (14) have been removed from erythrocytes by heating the cells at 56°C. Antibodies were demonstrated in the supernatant fluids by using the eluates to sensitize other erythrocytes. Several investigators (12, 15, 16) have similarly been able to elute the substance reacting with antiglobulin serum from the erythrocytes of patients with acquired hemolytic anemia. Heating the direct Coombs-positive, *in vivo* "sensitized" erythrocytes from case 2 destroyed their agglutinability in antiglobulin serum, but antibody could not be demonstrated in the eluate.

An attempt was then made to elute antibody from cells sensitized *in vitro*. Group 0 cells were chilled with complement in serum from case 2 and were shown to agglutinate in a 1–128 dilution of antiglobulin serum (indirect Coombs test [a]). After heating at 56°C for five minutes, these cells were weakly agglutinated by a 1–8 dilution of antiglobulin serum. Repeated attempts to demonstrate antibody in eluates from such normal cells sensitized in the serum from case 2 were inconclusive. Using serum from case 1 with a higher titer (4), however, antibody was adsorbed, eluted and transferred to other cells.

The basic technique mentioned above (12–16) was used. One ml. of packed group 0 cells was added to a mixture of 2.5 ml. of serum from case 1

TABLE II

The adsorption and elution of PCH antibody

Antibody in serum and eluate

		Dilution of serum or eluate							
		Undiluted	2	4	8	16	32	64	128
Serum									
Before adsorption	H*			+++	+++	++	+	0	0
	A†			++++	++++	++++	++	+	0
After adsorption	H*	0	0	0	0	0	0	0	0
	A†	0	0	0	0	0	0	0	0
Eluate	H*	+++	+++	++	++	0	0	0	0
	A†	++++	+++	+++	++	+	0	0	0

Antibody on erythrocytes‡

Erythrocytes	Dilution of antiglobulin serum						
	4	8	16	32	64	128	256
After adsorption	++++	++++	+++	++	+	+	0
After heating	++	++	+	0	0	0	0
Cells chilled in eluate	++++	+++	++	+	+	0	0

* Hemolysis in Donath-Landsteiner reaction
† Agglutination in indirect Coombs test (b)
‡ Agglutination in indirect Coombs test (a)

and 2.5 ml. of a 1–2 dilution of guinea pig complement. After standing in the ice bath for 30 minutes, the mixture was centrifuged. Titration of the serum showed that all of the antibody had been adsorbed (Table II). The erythrocytes were then washed six times with cold saline, and were found to be agglutinable in antiglobulin serum (Table II). The sensitized cells were added to 1.5 ml. of saline and heated, with frequent shaking, at 56°C for eight minutes. The indirect Coombs test (a) indicated that antibody had been removed from the surface of the erythrocytes. The supernatant fluid obtained after centrifuging the heated cells was then used in place of PCH serum in the Donath-Landsteiner and indirect Coombs tests (Table II). This eluate contained approximately one-fourth the antibody found in the original serum. By using a serum with a high antibody titer, therefore, it was possible to adsorb repeatedly PCH antibody to erythrocytes and then demonstrate antibody in an eluate obtained by heating the erythrocytes at 56°C. Such results were not obtained with normal serum examined in parallel with the PCH serum.

The effect of heat on both human and sheep erythrocytes was then studied. Human erythrocytes heated at 56°C for five to ten minutes were still capable of adsorbing antibody. When cells were held at this temperature for 15 minutes, many were destroyed, but the remaining cells adsorbed PCH antibody. Sheep cells so heated could still be sensitized by amboceptor. Sheep cells heated after sensitization were still hemolyzed when incubated with complement.

Comment: The demonstration that PCH antibody can be eluted from erythrocytes may aid in characterizing this antibody. Two facts made elution possible: *1*) the failure of the antibody to act as a hemolysin in the absence of complement and *2*) the stability of the antibody at 56°C. Experiences with sera from the two patients indicate that the PCH serum must have a high antibody titer if antibody is to be detected in the erythrocyte eluate. The mechanism of the dissociation of the erythrocyte-antibody complex is not clear. The effect of heat was apparently not that of "receptor" destruction. The antibody must fix with complement, and it may be that heat releases the antibody by inactivating complement. Other antibodies, however, that do not require

complement for fixation can also be eluted from erythrocytes by heating at 56°C (12–16).

Because antibody could not be demonstrated in eluates from cells sensitized *in vivo* or *in vitro* in the serum of case 2, the elution technique did not help to clarify the nature of the "cell antibody" detected in the direct Coombs test.

Identification of PCH serum factor reacting with antiglobulin serum as PCH hemolysin

The use of antiglobulin serum to detect and measure PCH antibody indicated that agglutination in the indirect Coombs tests was dependent on the same serum factor that produced hemolysis in the Donath-Landsteiner reaction (4). The indications that this is true are summarized as follows:

1) Neither Coombs antibody nor hemolysin was adsorbed at 10–12°C; both required 4°C for adsorption.

2) Coombs antibody and hemolysin were adsorbed simultaneously.

3) The titers of Coombs antibody and hemolysin were the same (4).

4) Both withstood heating at 62°C for 30 minutes.

5) There was a reciprocal relationship between the amount of complement present and both the amount of Coombs antibody fixed and the hemolysin titer (4).

6) Both, as will be indicated, are in the same serum fraction.

The separation and characterization of the PCH antibody

Characterization of PCH antibody necessitated separation of the antibody from other serum components. The possibility existed that the patient's serum might contribute C'1 and C'3 activity or an abnormal component which would complete the hemolytic reaction in the presence of C'2 and C'4. Fraction PI contained C'1,C'3, and C'4 but did not contain any PCH antibody (Table III). The precipitates formed when Fraction SI was dialyzed did not contain PCH antibody nor did they contain any of the components of complement. On the basis of these studies, which showed that the PCH antibody was not associated with C'1, C'3, and C'4, nor with the euglobulins which are insoluble between pH 5.0 and 7.0, the possible association of PCH antibody with C'2 or with the gamma globulins was next investigated.

The precipitates II + III, IV and V were examined for their PCH antibody content (Table III). II + III was the only fraction which contained PCH antibody. II + III prepared in the manner described above did not contain any of the known components of complement, and it was obvious that the PCH antibody was not associated with complement in human plasma.

TABLE III

Titration of PCH antibody in various fractions of serum

Serum fraction	Antibody titration										
	Hemolysin					Indirect Coombs (b)					
	Undiluted	2	4	8	16	Undiluted	2	4	8	16	32
Original plasma	+++	++	+	0	0	+++	++	++	0	0	0
PI*	0	0	0	0	0	0	0	0	0	0	0
II+III*†	0	+	++	+	0	+++	+++	+++	++	++	0
IV*	0	0	0	0	0	0	0	0	0	0	0
V*	0	0	0	0	0	0	0	0	0	0	0
II‡	+++	++	++	+	0	++++	++++	++++	+++	+	0
III–1‡	++	+	0	0	0	++++	+++	0	0	0	0
II E§	0	0	0	0	0	0	0	0	0	0	0
II S§	++	+	0	0	0	++++	+++	++	0	0	0

* Fraction concentrated five times with respect to original plasma.
† II+III anticomplementary in dilutions through 1–4.
‡ Fraction concentrated four times with respect to original plasma.
§ Fraction concentrated two and one-half times with respect to original plasma.

When II + III was separated into water soluble (II + IIIS) and water insoluble (II + IIIE) fractions, PCH antibody was found in both of these fractions. II + IIIS and II + IIIE were therefore combined (II + III) and fractionated into II and III − 1. Practically all of the PCH activity was found in Fraction II. Small amounts were found in III − 1. Electrophoretic analysis of Fraction II revealed that it was entirely homogeneous with a mobility identical to that of the gamma globulins.

When Fraction II was separated into water soluble (IIS) and water insoluble (IIE) fractions, all of the PCH antibody was found in IIS indicating that this antibody is associated with water soluble gamma globulins and resembles most other human antibodies in this respect (9).

Comment: It is apparent that none of the known components of complement are associated with PCH antibody. In fact, the antibody is a gamma globulin.

PCH antibody in Fraction II + III behaved as though it was a euglobulin but actually showed its true pseudoglobulin character in Fraction II. This discrepancy may be due to interactions occurring between PCH antibody and other globulins in II + III. The removal of alpha and beta globulins allowed the antibody to exhibit its solubility in water.

While most of the PCH antibody was present in II, small amounts were found in III − 1. The gamma globulin originally present in II + III was distributed in the proportion of 80 per cent in II and 20 per cent in III − 1. Thus, the activity found in III − 1 may be accounted for by the presence of small amounts of gamma globulin in this fraction. When Fraction II was separated into euglobulins and pseudoglobulins, all of the antibody activity was associated with the water soluble gamma globulins (IIS). Fraction IIS contains other antibodies as well as the PCH antibody (9), and it is hoped that PCH antibody can be separated from IIS by adsorption and elution. The possibility still exists that a serum factor independent of complement and antibody is concerned in the hemolysis of paroxysmal cold hemoglobinuria. Complete elucidation of this problem must await studies with pure components of complement and pure PCH antibody.

SUMMARY

Erythrocytes from a patient with paroxysmal cold hemoglobinuria (PCH) were found to be agglutinable in antiglobulin serum (direct Coombs test). Although these erythrocytes had been "sensitized" *in vivo*, they were not hemolyzed when warmed with complement. The cells, however, were hemolyzed, both *in vivo* when the patient was chilled, and *in vitro* when they were chilled and warmed in PCH serum.

The patient's cells were no longer agglutinable in antiglobulin serum six hours after *in vivo* hemolysis. No concomitant change in either serum antibody or complement level was detected. Further characterization of the "cell antibody" responsible for agglutination in the direct Coombs test was not possible since the factor could not be demonstrated in eluates from the patient's erythrocytes.

The behavior of the abnormal antibody present in the sera of two patients with PCH was studied by using antiglobulin serum (indirect Coombs test) as well as the Donath-Landsteiner reaction. Antibody adsorbed *in vitro* from serum with a high antibody titer could be eluted from erythrocytes by heating the cells at 56°C.

The PCH serum factor which reacts with antiglobulin serum was identified as the PCH hemolysin.

Fractionation of PCH serum in alcohol-water systems revealed that the PCH antibody is a water soluble (pseudoglobulin) gamma globulin and resembles most other human antibodies in this respect.

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DIRECT ACTION OF VITAMIN B_{12} UPON HUMAN BONE MARROW

THE EFFECT OF INSTILLATIONS OF VITAMIN B_{12} AND FOLIC ACID INTO THE BONE MARROW AS STUDIED BY NUCLEIC ACID STAINING TECHNIQUES [1, 2]

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Folic acid and vitamin B_{12} are involved in chemical reactions which lead to the formation of nucleic acids in living cells. These relationships are amply demonstrated by studies in bacterial (1–5), animal (6), and human (7) metabolism. It appears likely that folic acid is essential to the formation of various purines and pyrimidines such as thymine from endogenous sources of carbon and amino nitrogen as well as to the interconversion of these substances or of their ribosides (8). Vitamin B_{12}, on the other hand, appears to be active in the formation of ribosides, such as thymidine, from these purines and pyrimidines. Folic acid and vitamin B_{12}, therefore, appear to be active at different stages of a chemical chain reaction which leads to the formation of nucleic acids.

In this paper, we wish to report a method of direct instillation of test substances into human bone marrow cavities. By such a method, observations may be made on the effect of various substances to be tested on marrow cells in their natural environment. By the use of this method, evidence has been obtained that vitamin B_{12} can be utilized unchanged by the megaloblasts and early erythroblasts of patients with pernicious anemia in relapse. On the other hand, folic acid has no such direct effect. Methyl green-pyronin stain applied to the erythroblasts of patients with pernicious anemia in relapse before and after the instillation of vitamin B_{12} into the bone marrow indicates that vitamin B_{12} corrects a qualitative abnormality of the cytoplasmic ribonucleic acid of these cells. Folic acid has a similar effect only after oral or parenteral administration and is presumably activated by enzymatic activity elsewhere in the body. These data offer additional evidence relating vitamin B_{12} and folic acid to nucleic acid metabolism.

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[2] Read in part before the Forty-second Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N.J., May 1, 1950.

MATERIAL AND METHODS

Injections into the marrow cavities of the iliac crests of six persons with pernicious anemia in relapse were performed as follows: three received 1 microgram of crystalline vitamin B_{12} [3] and three received 1 or 2 mgs. of folic acid.[4] In each instance, the material was injected slowly in 1 c.c. of solution after aspiration of 2–3 c.c. of marrow and blood. Moderate pain accompanied the injection and was diminished by slowing the rate of instillation. Forty-eight hours later, bone marrow was again aspirated from the exact site of previous instillation and also from the opposite iliac crest. The site of instillation was easily located by using the trauma produced by the earlier puncture as a landmark. In one case, marrow was aspirated also from sites 4 and 8 centimeters distant from the site of instillation of the vitamin B_{12}. Coverslip preparations were made of each marrow specimen obtained and stained *1*) with Wright-Giemsa stain for differential cell counts, *2*) with methyl green and pyronin B (9) for demonstration of cytoplasmic ribonucleic acid, and *3*) by a Stowell's modification of the Feulgen method (10) for demonstration of desoxyribonucleic acid. The methyl green-pyronin stain was prepared by mixing three parts of a 1 per cent solution of methyl green [5] containing 0.25 per cent liquefied phenol

[3] The material used was "Cobione," supplied by Merck and Co., Inc., Rahway, N.J., and provided in 1 c.c. ampules containing 10 micrograms of crystalline vitamine B_{12} per c.c. Appropriate dilutions were made for this study with physiologic saline solution.

[4] The material used was "Folvite," supplied as a powder by the Lederle division of the American Cyanamid Corp., Pearl River, N.Y. Solutions containing 1 or 2 mgs. of folic acid per c.c. were made in distilled water containing sodium bicarbonate sufficient for a pH of approximately 7.5.

[5] Methyl green and pyronin B were obtained from the National Aniline Division, Allied Chemical and Dye Corp., New York, N.Y. The stains used were certified by the Biological Stain Commission.

TABLE I

Nucleated erythroid cells in bone marrow before and after local instillation of vitamin B_{12}

		Case I			Case II			Case III		
		Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}	Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}	Before vit. B_{12}	After vit. B_{12}	Opposite ilium after vit. B_{12}
			1 gamma			*1 gamma*			*1 gamma*	
Megaloblasts	Per 100 WBC	16.0	3.5	11.0	13.5	1.5	17.5	15.5	3.0	13.0
Early erythroblasts		32.0	15.5	32.5	26.5	26.5	28.5	35.5	26.5	37.5
Late erythroblasts		18.0	25.5	14.5	15.0	35.0	18.5	12.0	28.0	20.5
Normoblasts		43.0	100.5	43.5	40.5	90.5	48.0	24.5	87.5	21.5

to one part of a similarly prepared solution of pyronin B.[5] Coverslip marrow films were stained for ten minutes following fixation for one minute in 95 per cent alcohol.

Films of bone marrow from a patient with erythroid hyperplasia due to chronic blood loss and from a normal person were treated in the same manner and served as controls. The red color of the cytoplasm produced by the pyronin stain indicated the presence of ribonucleic acid since this color could not be developed following incubation of films for three hours at 60° C. in a 0.1 per cent solution of crystalline ribonuclease[6] buffered with sodium barbital to a pH of 6.75 (11).

RESULTS

Effect of local marrow instillation of vitamin B_{12} on erythrocyte maturation: Table I is a chart of differential counts of nucleated cells of the erythroid series in bone marrow from three patients with pernicious anemia in relapse before and after the instillation of vitamin B_{12} into the marrow cavity. Stimulation of erythroid maturation is shown in all three instances by a decrease in the number of megaloblasts and an increase in normoblasts in the specimens obtained 48 hours after the local marrow instillation of the Vitamin B_{12} (see Figures 1 and 2). The marrow obtained from the opposite iliac crest was unaffected, indicating that the stimulation induced by vitamin B_{12} was local when the amount instilled was 1 microgram. However, the local marrow instillations of 15 micrograms resulted in a systemic response. In the one instance that marrow was aspirated 4 and

[6] Crystalline ribonuclease was provided by the Worthington Biochemical Laboratory, Freehold, N. J.

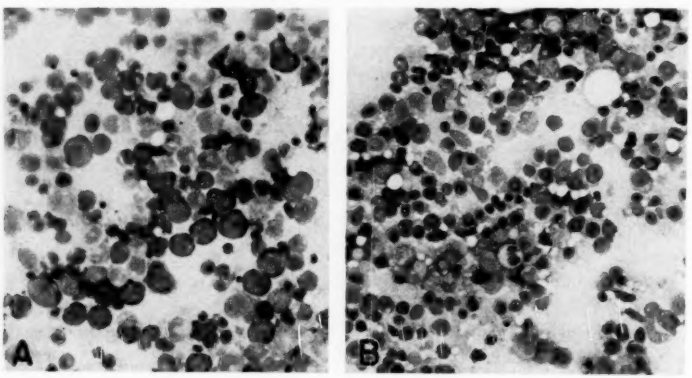

FIG. 1. THE EFFECT OF LOCAL MARROW INSTILLATION OF VITAMIN B_{12} ON WRIGHT-GIEMSA STAINED MARROW FILMS

A megaloblastic maturation arrest characteristic of pernicious anemia is seen in (A). A shift to a normoblastic maturation level at the injection site 48 hours after instillation of vitamin B_{12} is seen in (B). At this time, the marrow obtained from the opposite ilium was identical with (A). (× 320)

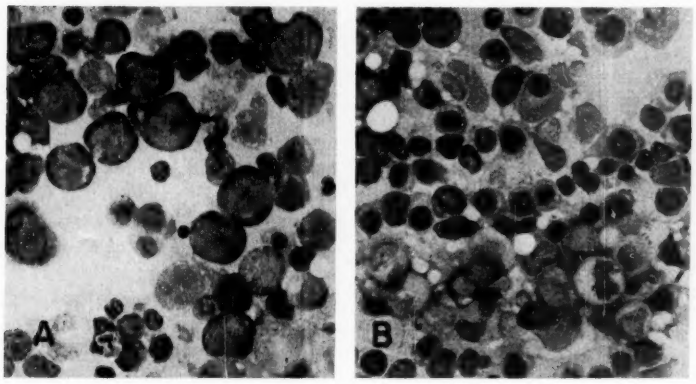

FIG. 2. MAGNIFICATION OF CELLS FROM FIGURE 1

(A) Megaloblasts of pernicious anemia. (B) Normoblasts. Wright-Giemsa stain. (× 650)

8 centimeters from the site of injection, the stimulating effect decreased as the distance increased.

Effect of local marrow instillation of folic acid on erythrocyte maturation: Table II is a chart of differential counts of nucleated erythroid cells in bone marrow from three patients with pernicious anemia in relapse before and after the local marrow instillation of 1 or 2 mgs. of folic acid. It is apparent that there was no stimulation to erythroid maturation at the site of injection of folic acid. The counts done on marrow before and after injection and on marrow from the opposite ilium are all similar. Furthermore, no change in the appearance of Wright-Giemsa stained marrow films was noted after instillation of folic acid into the marrow cavity.

Effect of local marrow instillation of vitamin B_{12} and folic acid on the nucleic acid content of erythroid cells: The character and distribution of ribonucleic acid in the cytoplasm of young erythroid cells were observed in methyl green-pyronin stained preparations of bone marrow from a patient with erythroid hyperplasia resulting from anemia of chronic blood loss. Study of such a preparation (Figure 3) reveals a diffuse and homogeneous distribution of the red granules throughout the cytoplasm of the early erythroblasts. In the later forms, they seem to become more condensed and clumped. Finally, during the normoblastic stage of the development of the erythrocyte, the red color, indicating cytoplasmic ribonucleic acid, disappears altogether.

Methyl green-pyronin stained marrow preparations from patients with pernicious anemia in relapse, one of which is presented in Figure 4-A, show that the distribution of the cytoplasmic ribonucleic acid is quite different in this disease. In these preparations, there is condensation and

TABLE II

Nucleated erythroid cells in bone marrow before and after local instillation of folic acid

	Case IV			Case V			Case VI		
	Before folic acid	After folic acid	Opposite ilium after folic acid	Before folic acid	After folic acid	Opposite ilium after folic acid	Before folic acid	After folic acid	Opposite ilium after folic acid
		1 mg.			*1 mg.*			*2 mgs.*	
Megaloblasts	6.0	7.0	3.5	7.5	8.5	5.5	13.5	15.0	11.0
Early erythroblasts (Per 100 WBC)	10.5	8.0	6.5	25.5	23.0	16.5	22.0	19.0	18.5
Late erythroblasts (Per 100 WBC)	7.0	3.0	4.5	17.5	21.5	16.0	15.0	12.0	10.5
Normoblasts (Per 100 WBC)	24.5	17.5	27.0	21.5	29.0	31.0	20.0	25.0	27.5

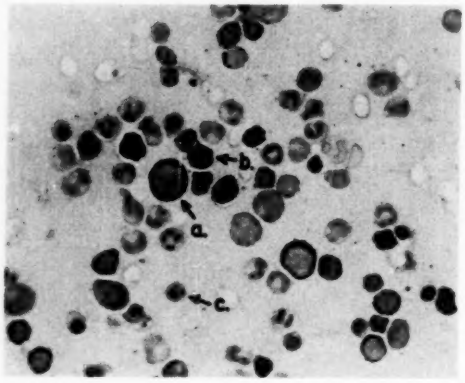

FIG. 3. A FILM OF MARROW FROM A PATIENT WITH CHRONIC BLOOD LOSS ANEMIA STAINED WITH METHYL GREEN AND PYRONIN B

Cytoplasmic ribonucleic acid stains red; nuclear structures stain green. a = early erythroblast; b = late erythroblast; c = normoblast. (× 650)

clumping of the red granules in the cytoplasm of the young cells of the erythroid series. In similarly stained marrow smears (Figure 4-B) after local marrow instillation of vitamin B_{12}, there is a change in the appearance of the cytoplasmic ribonucleic acid of these early erythroid cells. It becomes more diffuse and homogeneous; and, in general, it assumes the characteristics of that observed in early erythroblasts from a patient with anemia due to chronic blood loss.

No change in the clumped cytoplasmic ribonucleic acid was noted 48 hours after the local marrow instillation of folic acid. However, when folic acid was given orally or intramuscularly in therapeutic doses, the cytoplasmic ribonucleic acid became homogeneous and of more normal appearance.

The appearance of desoxyribonucleic acid stained by Stowell's modification of the Feulgen method was not altered in the preparations obtained after the instillation either of vitamin B_{12} or of folic acid. Furthermore, the desoxyribonucleic acid in erythroblasts from persons with pernicious anemia in relapse had the same appearance as that from a person with anemia of chronic blood loss.

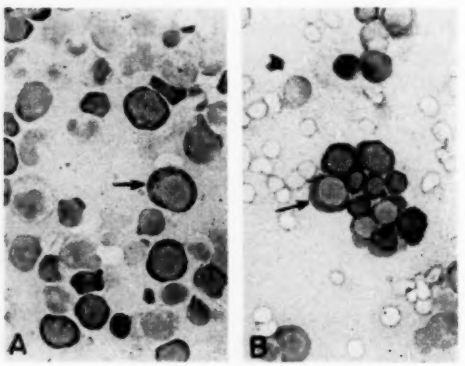

FIG. 4. THE EFFECT OF LOCAL MARROW INSTILLATION OF VITAMIN B_{12} ON METHYL GREEN-PYRONIN B STAINED MARROW FILMS

In (A) arrow points to early erythroblast in marrow from patient with pernicious anemia in relapse. In (B) arrow points to early erythroblast in marrow obtained 48 hours later from the injection site of vitamin B_{12}. (× 650)

DISCUSSION

The normal maturation of erythroblasts in the bone marrow of persons with pernicious anemia in relapse observed at the site of instillation of vitamin B_{12} but not in marrow from the opposite ilium demonstrates that vitamin B_{12} can be utilized directly by bone marrow cells without alteration by any other organ or tissue. Therefore, if vitamin B_{12} and extrinsic factor of food are the same substance, as seems probable, this experiment provides direct evidence that extrinsic factor need not be chemically changed by intrinsic factor or by the liver before becoming active for erythrocyte maturation. This, of course, is not a new concept. Studies of Berk and his associates (12) on hematologic responses in persons with pernicious anemia, who received vitamin B_{12} and refined liver extract orally along with normal human gastric juice, suggest that vitamin B_{12} (presumably extrinsic factor of food) and the antipernicious anemia principle of liver are identical or closely related compounds. After observing the magnitude of fecal elimination of vitamin B_{12} in patients with untreated pernicious anemia and the hematologic responses in similar patients obtained by the administration of 10 per cent alcoholic extracts of beef muscle, they concluded that ". . . the function of the intrinsic factor of normal human gastric juice is to facilitate the absorption

by the intestine of vitamin B_{12} . . . rather than to react with the extrinsic factor as hitherto assumed."

On the other hand, in our experiment, folic acid was not utilized unchanged within 48 hours after instillation of the substance into the marrow cavity. Observations by others provide further data that folic acid, acting as a substrate, in some way must be altered before becoming active in hematopoiesis. Norris and Majnarich have reported that folic acid has no effect on hematopoiesis as observed by an *in vitro* rabbit bone marrow culture technique (13). However, after this substance was incubated with milk xanthine oxidase, liver homogenate, or extract of rat gastric mucosa, it became hematopoietically active as demonstrated by this method (14). Tove and Elvejhem (15) have reported the presence of a substance in methanol extract of liver which has a sparing effect on folic acid in the metabolism of mink. Furthermore, this substance protected these animals from folic acid deficiency produced by feeding them 7-methyl folic acid.

Further stimulation to the study of the mode of action of folic acid in hematopoiesis was provided by the concentration by Sauberlich (16) of a substance in rat urine which is a growth factor for *Leuconostoc citrovorum* 8081. Studies on this factor, termed the "citrovorum factor," have demonstrated that *1*) the urinary excretion of the substance is increased by oral administration of folic acid both in rats (17) and in human beings (17,18), and *2*) the substance counteracts the toxicity of aminopterin as measured by growth of *L. citrovorum* (17). Data are not yet available on the hematopoietic activity of this substance in erythrocyte maturation factor deficiency anemia. Preliminary studies in this laboratory indicate that the local marrow instillation of four million units of "citrovorum factor" does not result in stimulation of erythrocyte maturation in persons with pernicious anemia in relapse.

The changes observed in the cytoplasmic ribonucleic acid of erythroid precursor cells in marrow from subjects with pernicious anemia in relapse as compared with those from persons with anemia of chronic blood loss provide data linking erythrocyte maturation factor deficiency anemia to abnormalities in nucleoprotein metabolism. Thorell, in 1947 (19), correlated, by cytochemical and ultraviolet microphotographic studies, endocellular growth process in hematopoiesis with the presence of ribonucleic acid in the granulocytic and erythroid precursor cells in bone marrow. Briefly, he found that growth and new cellular protein formation during hematopoiesis are associated with high concentrations of ribonucleic acid in the cytoplasm and the nucleolus of these cells; as growth activity declines, the concentrations of ribonucleic acid decrease proportionately. In normal erythropoiesis, hemoglobin synthesis does not begin until the ribonucleic acid metabolism is completed; after this, the synthesis of hemoglobin proceeds rapidly. In pernicious anemia, there is an abnormality in these relationships of growth and differentiation; in this disease, hemoglobin synthesis begins while the ribose polynucleotide content of the cell is still high. The apparent correction of this abnormality observed in our study after the local marrow instillation of vitamin B_{12} provides additional data linking vitamin B_{12} and nucleoprotein metabolism.

SUMMARY AND CONCLUSIONS

1. A method of direct instillation of test substances into human bone marrow cavities is described.

2. Vitamin B_{12} can be utilized locally by the bone marrow cells and corrects a qualitative abnormality in cellular ribonucleic acid in persons with pernicious anemia in relapse. It need not be altered by stomach or liver to exert this effect.

3. Folic acid is not utilized locally by bone marrow cells within 48 hours of instillation into the marrow cavity; but when given orally or parenterally, it has the same cytologic and cytochemical effects as vitamin B_{12}. It is probable that folic acid must be converted to an active hematopoietic substance by enzymatic activity elsewhere in the body.

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NEURAL MECHANISMS INVOLVED IN ITCH, "ITCHY SKIN," AND TICKLE SENSATIONS

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Available evidence suggests that the sensation of itching is closely related to that of pain (1). Titchener (2) observed that when the skin was explored with a fine hair, well-defined points were found which gave rise to itching when the intensity of stimulation was low, and to pain on stronger stimulation. Bishop (3) found that itching resulted from repetitive low intensity electrical stimulation of pain spots in the skin. Lewis, Grant and Marvin (4) pointed out that noxious stimuli, if their intensity be decreased, can be made to produce itching instead of pain. Förster (5) and Bickford (6) reported that in patients who had undergone section of the lateral spinothalamic tracts, thus abolishing perception of cutaneous pain but not of touch, itch was also abolished. Bickford also observed this combination of sensory changes in two patients with syringomyelia. Ehrenwald and Königstein (7), however, stated that they had found two cases of syringomyelia with loss of pain but preservation of touch and itch, and one in which pain was intact but touch and itch absent. McMurray (8) and Kunkle and Chapman (9) found that individuals who were constitutionally insensitive to pain were likewise insensitive to itching, but they experienced touch sensation. Bickford observed that itching and pain disappeared at the same time when a cutaneous nerve was anesthetized. Hardy, Wolff and Goodell (10) observed that in addition to feelings of warmth, occasional itching resulted from sustained thermal irradiation at an intensity not quite strong enough to induce pain. Thöle (11) found during spinal anaesthesia, as the sensitivity of the skin to painful stimuli decreased, that stimuli which initially induced pain later induced itching and finally no sensation at all.

In the present investigation many of the experiments described by Goldscheider (12), Bickford, and Lewis and associates have been repeated. Additional experiments were designed to illuminate further the neural mechanisms involved in itch, "itchy skin" and tickle sensation.

ITCH

Subjects and methods

The subjects were healthy adults, chiefly the authors, although other individuals participated from time to time. Itching was elicited by the application of cowhage to an area of skin approximately 1 cm. in diameter. Cowhage is the familiar "itch powder," consisting of the fine fibers or spicules of the plant *Mucuna pruriens*. It was found by trial that the itch so produced was indistinguishable from that following the bites of mosquitoes, or the intracutaneous injection of histamine. When an area of skin on the arm, hand, leg or back was used for experimental procedures the corresponding areas on the opposite side of the body served as control.

Description of sensation induced following application of cowhage to the skin

Cowhage applied to an area of skin approximately 1 cm. in diameter induced, usually within 10–20 seconds, an intense itching sensation which had both burning and pricking qualities. It was localized but seemed to spread for a few millimeters beyond the borders of the stimulated area. These itch sensations sometimes merged into pain of a burning and pricking quality. The skin under the cowhage rapidly became red, and a flare sometimes spread into adjacent areas of skin. When the area involved was the volar surface of the forearm, the flare spread as far as 2 cm. proximally and distally and approximately ½ to 1 cm. laterally. Within a few minutes after application of the cowhage, the skin several centimeters proximally and distally around the itching area became hypoalgesic to pin prick, and around the edge of this zone was a narrow band of hyperalgesia to pin prick, approximately ½ cm. wide. The itching from cowhage spontaneously diminished after a few minutes and did not return until the cowhage was mechanically agitated; or until it was re-

vived by stroking the surrounding skin with the finger tip or a blunt object.

When the cowhage applied to the skin produced an intense itching, the sensation sometimes eventually became indistinguishable from pain. Furthermore, occasionally cowhage on normal skin and in areas of primary or secondary hyperalgesia induced pain alone. Also, as mentioned above, a heat stimulus at an intensity just below that sufficient to elicit pain, elicited itching.

Peripheral Fiber Pathways Involved in Itch Sensation

It has been demonstrated that stimulation of the human skin with a pin may give rise to two kinds of painful sensation. The first pain to be perceived after the application of the pin is described as "pricking" and is punctate, superficial and well localized. This has been referred to as "first" or "fast" pain. There is also a second, different type of pain, described as "burning," which is perceived as more diffuse and less superficial than the "pricking" pain. Since this "burning" pain is perceived after a slightly but definitely longer latent period than the "pricking" pain, it has been referred to as "second" or "slow" pain (13, 14). Although Lewis denied that these experiences constituted different qualities of cutaneous pain, others have recognized the qualitative difference. Most individuals can readily distinguish between the "pricking" and "burning" in cutaneous pain (15), especially if they are separately presented by the experimental methods described below.

The work of both Lewis and co-workers and Gasser and co-workers suggests that the "fast" pain is a function of myelinated fibers, the "slow" pain of unmyelinated ones (14).

Experiment 1. Demonstration of an itching sensation with pricking quality

In three subjects procaine hydrochloride 1% was infiltrated about a cutaneous nerve on the ventral aspect of the forearm. Within a few minutes there developed distal to the site of infiltration an area in which pin prick elicited only a sharp, superficial, well-localized pain of short latency—the "first" pain. The slower, diffuse and burning "second" pain was not felt.

Within this area of altered sensibility cowhage elicited itching which was sharp, pricking, superficial, and readily localized. There was no burning component. In areas where there was complete absence of pain sensitivity, but where light touch was still present, itching was not felt.

Experiment 2. Demonstration of itching sensation with a burning quality

a) In a series of six experiments on four subjects ischemia of the forearm was obtained by a pressure of 200 mm. Hg maintained around the upper arm by means of a sphygmomanometer cuff. After about 20 minutes of ischemia in the arm, a pin prick applied to the fingers and the hand elicited pain of long latency which was poorly localized, and burning in character. The "first" pain was absent, as was light touch.

Within these areas of altered sensibility the application of cowhage on a site approximately 1 cm. in diameter on the back of the hand resulted in itching which was different from that obtained in Experiment 1, in that it was diffuse and poorly localized, seemed to be a little distance beneath the surface of the skin, and was "burning" in quality.

However, in more proximal areas of the ischemic forearm, closer to the occluding cuff, where normal pain and touch sensation were retained, cowhage produced itching no different from that in skin of the opposite arm with blood and nerve supply intact.

b) The course of a branch of a superficial cutaneous nerve in the forearm was mapped through a distance of 4–6 cm. by means of faradic stimulation in two subjects. A soft lead tubing was applied over the nerve and held in place by tapes. Brine at a temperature of $-2°$ to $-4°$ C was circulated through it for 30 minutes, resulting in the progressive anesthetization of the area supplied by the nerve (16).

After 30 minutes, within this area of altered sensation "second" pain could be elicited by a pin point but "first" pain and light touch were absent. In these areas "burning" itch could be elicited by cowhage but not "pricking" itch.

Comment

It was thus possible to separate the usual itch sensation, as it occurs after insect bites or the application of cowhage, into two components. These corresponded in quality to the two types of cutaneous pain, and the results of differential blocking of cutaneous nerves indicate that they are mediated by different fibers. It is suggested that "pricking" itch is carried by the myelinated fibers responsible for "first" pain, and that "burning" itch is carried by unmyelinated fibers responsible for the "second" pain. Since either kind of itch could occur in skin in which touch perception was absent, it seems probable that touch receptors and nerves are not involved in the perception of itching.

It might be objected that the sensory changes following obstruction of the circulation are due to interference of fluid movement which might modify the function of sensory end organs, rather than to any functional change in the nerves themselves.

Against this assumption is the evidence that normal sensation was retained in skin close to the occluding cuff, but still distal to the obstruction, where fluid movement was as much interfered with as in the more distal areas where sensory changes did occur.

It is not difficult, once the two sensory components of itching have been separately perceived, to identify them in spontaneously occurring itches or in itch induced by a heat stimulus, by intracutaneous puncture of histamine or by exposing the arm to multiple mosquito bites.

Since the sensations of itching and pain are apparently mediated by the same fiber pathways an attempt was made to ascertain in what way a stimulus which produces itching differs from one which produces pain. Hence, experiments to test the thesis that the stimuli are of the same kind but of lower intensity were designed.

Experiment 3. Demonstration of lowered pain threshold in itching areas

Several areas of skin of the volar surface of the forearm were blackened with India ink, as shown in Figure 1. Pain thresholds in all areas were measured by the thermal radiation method of Hardy, Wolff and Goodell (17). Cowhage was applied to the central area in the usual way. Pain thresholds were then measured repeatedly both when itching was present and in the intervals when it had spontaneously but temporarily ceased.

It was found that the pain threshold in all five areas was the same before cowhage was applied (190 ± 5 millicalories/sec./cm.^{2}). After itching started in the central area, however, the threshold in this zone was lowered (120 to 160 mc./sec./cm.^{2}), the lowest thresholds being found at the times of highest itch intensity. When itching temporarily ceased, the threshold returned to its initial level, and was lowered again on the resumption of itching. In the other areas the threshold remained constant or was slightly elevated to 210 ± 10 mc./sec./cm.^{2} when the central area was itching. These observations were made in three series of experiments in each of two subjects. The application of the heat stimulus to the central area in itch free intervals was usually followed by recrudescence of the itching.

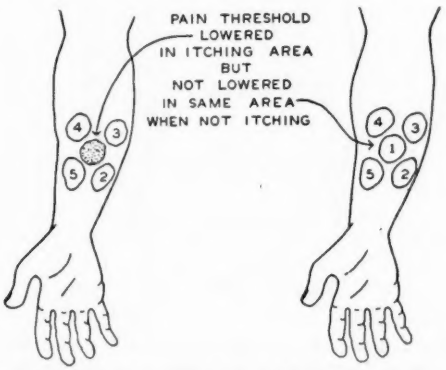

FIG. 1. LOWERING OF THE PAIN THRESHOLD IN AN AREA OF ITCHING SKIN

Cowhage was applied only to area 1. When itching occurred pain threshold was lowered in area 1, but not in adjacent areas 2, 3, 4, and 5.

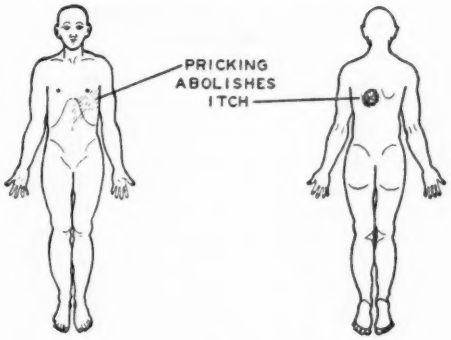

FIG. 2

Itching induced on the back by cowhage was abolished by painful pin pricks in the same dermatome on the anterior chest wall.

Comment

These observations are consistent with the view that stimuli which give rise to itching activate pain endings in the skin at a stimulation intensity below the pain threshold. Less additional thermal energy is therefore required to produce definite pain sensation if itching is already occurring. Hence the pain threshold as measured is lowered.

Alterations in Central Excitatory Processes Relevant to Itching

Experiment 4. Demonstration that itch can be abolished by painful pin pricks in adjacent skin

It was found in 20 subjects that when itching occurred spontaneously or when it was induced by cowhage, a pin pricked lightly several times in the zone of itching on the surrounding skin abolished the itching sometimes for long intervals. It usually returned after periods up to several minutes in duration, although the slight pain from the prick had completely faded within 10 to 15 seconds. This has been observed not only with itching due to cowhage, but also in the itching resulting from

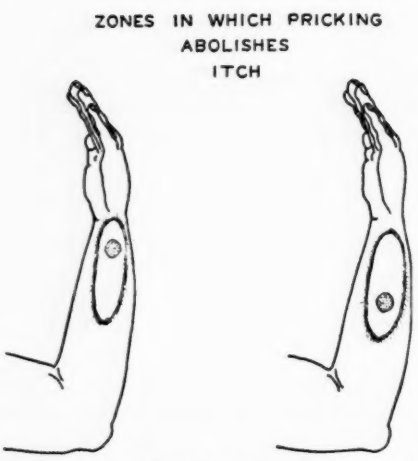

FIG. 3. ZONES IN WHICH PAINFUL PIN PRICKS ABOLISHED ITCHING INDUCED BY COWHAGE ON THE MEDIAL ASPECT OF THE FOREARM

Sites of cowhage application are indicated by stippling. The zones were surrounded by a narrow band, as indicated, of hyperalgesia.

insect bites, intracutaneous histamine puncture and intracutaneous foreign protein.

Pin prick applied not only locally but at considerable distance from the zone of itching abolished itching. With three subjects it was found that, if the cowhage was applied to the back, pin prick near the sternum in the same dermatome as the cowhage (Figure 2) was fully effective in eliminating itch.

In two subjects an attempt to delimit exactly the area within which pin prick abolished itching on the forearm was undertaken. Cowhage was applied at approximately the mid portion of the medial aspect of the forearm. After application of the cowhage the size of the area within which this effect could be demonstrated gradually increased and reached the eventual limit in about one hour following the beginning of itch. At this time the area measured approximately 5×15 cm. Figure 3 illustrates the area as mapped on the forearm of one subject on whom cowhage was applied as shown. It was essentially the same for the second subject.

After the mapping had been completed, cowhage was applied at the distal end of the area. The effect of pin prick was again tested, and it was found that the area within which it abolished itching was approximately the same as in the first instance, extending only 1–2 cm. further distally. Around this zone was a band of hyperalgesia to pin prick about 4 mm. wide.

A similar experiment was carried out on the legs of three subjects (Figure 4). The cowhage was applied just below the knee on the outer aspect of the left leg. Again there was an area within which the pin prick was effective in abolishing itch. After 60 minutes the area extended 7 cm. proximally and 24 cm. distally to the itching spot. On the leg, also, there was only slight difference in the area of effectiveness of the pin prick if the cowhage was shifted to another point within this area. A narrow band of hyperalgesia also surrounded this zone on the leg.

Comment

Scratching is known to relieve itch. It has been held (1) that the relief so obtained resulted from replacement of the itch with frank pain. In the above observations the painful stimulus of the pin prick was not only brief but also far removed, as much as 24 cm. on the leg or back from the itching area, yet the itching sensation could thus be obliterated. Furthermore, the itch did not return until some time after the pain from the prick had subsided.

These observations seem most readily explicable mainly in terms of central rather than peripheral processes, as will be discussed below. The areas mapped on the forearm fall within the limit of the first thoracic dermatome; those on the leg correspond closely to L–4 or L–5 as determined by Förster (5).

Experiment 5. Demonstration that a zone of secondary hyperalgesia is "anti-pruritic"

A superficial branch of a cutaneous nerve on the volar surface of the forearm was located by means of

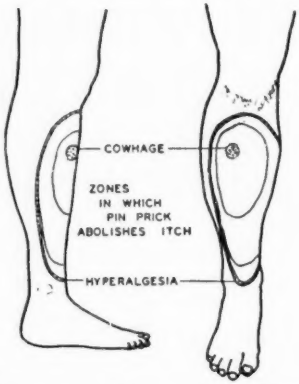

FIG. 4. ZONE ON THE LEG IN WHICH PAINFUL PIN PRICK ABOLISHED ITCHING INDUCED BY COWHAGE

The inner line indicates the extent of the zone after 15 minutes of itching; the outer border is its extent at the end of an hour. Sites of cowhage application are indicated by stippling. The zone was surrounded by a narrow band of hyperalgesia.

faradic current, and then was painfully stimulated for two minutes. Following this procedure there was established within 20 minutes in the surrounding skin a zone of "secondary hyperalgesia." In this zone of hyperalgesic skin the pain threshold as ascertained by the thermal radiation method was not lowered (10) but stimuli at threshold or above were perceived as more painful and longer lasting than stimuli of equal intensity in the surrounding skin.

Cowhage was applied in such an area of secondary hyperalgesia in four subjects in 11 observations. It evoked either occasional bursts of pain or no sensation at all, but not itching.

The secondary hyperalgesia was abolished in two subjects by infiltrating procaine at the site of injury by faradization and by vigorous pin pricks in a zone within 1–2 cm. around the point of faradization. Cowhage was again applied and itching now occurred as it did in control areas on the other arm.

In 12 other experiments on two subjects, areas of secondary hyperalgesia were induced by painfully pinching with forceps a small fold of skin on the volar surface of the forearm. Approximately 10 minutes after the pinch, when the secondary hyperalgesia was well developed, cowhage when applied failed to elicit itching. When the cowhage was pressed into the skin, a maneuver which intensifies itching in control non-hyperalgesic skin, pricking pain was experienced, but itch did not ensue.

Comment

Lewis, Grant and Marvin (4) observed that faradic stimulation of a cutaneous nerve was followed by a state in adjacent skin in which itching did not occur after intracutaneous histamine puncture. Bickford (6) called this state "anti-pruritic" and found that it also occurred after other forms of noxious stimulation, such as a sharp blow, a gnat bite, burn or freeze. These authors, however, did not investigate the relation of hyperalgesia to the anti-pruritic state.

The observations on the effect of pin prick in abolishing itch, and on the failure of itching to occur in areas of secondary hyperalgesia, seem to indicate the existence of events of a special sort in the central nervous system. Similarity of the size and shape of the affected areas to the dermatome suggests that the spinal cord is the site of such processes. Bickford also concluded that the "anti-pruritic" state must depend on some spinal cord mechanism.

Discussion

It has been held (1) that itching is a "protopathic," as opposed to an "epicritic" sensation. In these terms, the "first" itch might be considered epicritic, since it is readily localized, the second as protopathic since it is more diffuse. It is not clear, however, that any useful purpose is served by retaining this terminology.

The use of cowhage as a stimulus has been criticized on the grounds that the itching which results is impure (6). Histamine has therefore often been employed, but it was found that the itching which resulted was not nearly so intense as that produced by cowhage, and seemed to be chiefly of the "second" type. The "impurity" of the cowhage-provoked itching may well be simply the result of the simultaneous presence of both types of itch.

The objection might be raised that one of the two kinds of itch described above was not really itch, but was, instead, pain. The ultimate appeal in such a case must be to the introspection of the person experiencing the sensation, and the subjects in this investigation agreed that there were two kindred but distinguishable sensations which were both felt as "itch." They had in common their association with an urge to scratch the involved skin. Brack (18) also referred to two qualitatively different itch experiences.

It seems likely, in view of all the available data, that stimuli which produce itching initiate impulses which, after traversing the peripheral pain nerves, pass up the spinothalamic tracts of the cord. Since painful stimuli in general can be made to produce itching by lowering their intensity, it is reasonable to suppose that the difference between a painful and an itch-producing stimulus is one of intensity. The reduction in the pain threshold at times when itching is being experienced is further evidence in support of this view. The probable reason for the lowered threshold is that the peripheral pain endings are already partially activated by the cowhage, so that the radiant energy which must be applied to give pain is less than that necessary if they have not been stimulated at all.

Potelunas, Meixner and Hardy (19) reported that there was no consistent difference in the cutaneous pain thresholds of patients with itching dermatoses as opposed to healthy individuals. As they pointed out, however, structural changes such as thickening had often occurred in the diseased skin, with consequent alterations in pain sensitivity. All of the observations in the present investigations have been made on skin of subjects

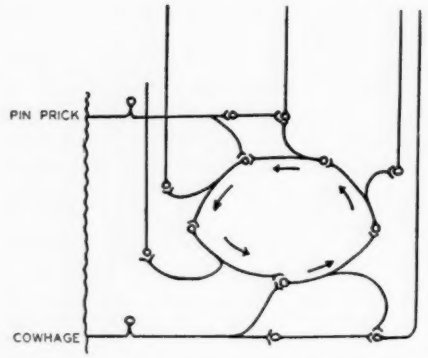

FIG. 5. SUGGESTED ARRANGEMENT OF CIRCUITS IN INTERNUNCIAL NEURONS RESPONSIBLE FOR ITCHING
Painful pin prick presumably breaks up the circuits.

without skin disorders, and without alteration of any sort except by the test procedures themselves. It must be emphasized that the threshold was found to be lowered only when the skin was itching.

One thesis which will explain all the findings is that the perception of itching depends on the presence in the cord of a circuit of internuncial neurons, analogous to those described by Lorente de No (20), around which impulses are constantly travelling. According to the hypothesis the establishment of such a circuit is the result of the low discharge frequency in a peripheral nerve which is weakly stimulated. As the circuit is traversed, discharge to spinothalamic neurons occurs, and hence impulses in the spinothalamic tract are sent upward to the brain in an orderly pattern (Figure 5). We suppose that the presence of this pattern of discharge in the brain is a necessary condition for the perception of itching. It is of interest that recently one subject following frontal lobotomy had an unaltered appreciation of itch, tickle and "itchy skin."

Such a circuit of excitation in an internuncial system of neurons would, of course, require a rather delicate adjustment of impulse frequencies and refractory periods. A pin prick in the dermatome in which itch is arising would, it is assumed, bring about a diffuse discharge in the corresponding cord segment, ramifying along many of the internuncial neurons involved in the itch circuit. The orderly pattern would therefore be temporarily destroyed, and itching halted. It would presumably require an appreciable time for it to be re-established, a time represented by the interval after pin pricking before itching is again perceived.

Hardy, Wolff and Goodell (10) have concluded that secondary hyperalgesia in the skin adjacent to a source of noxious impulses, results from the presence of an augmented central excitatory state in internuncial neurons. Such a state is indicated by the stipplings in Figure 6. It presumably acts to facilitate the passage of impulses at synapses. When cowhage is applied to skin in which such hyperalgesia is present, it may be supposed that the resulting impulses cannot set up the usual orderly circuits in the cord which result in itch sensation. The occasional burst of impulses elicited by the cowhage which is intense enough to cross the pain threshold of the pathway involved brings about a widespread discharge of the excitatory state and pain alone is experienced.

Such an explanation may outrun the data currently available about events in the human spinal cord, but on the other hand it does not do violence to any of the known facts of neural activity. The "all-or-none" law of impulse size in single nerve fibers does not come into question in this connection since differences in stimulus intensity, experienced as graded intensities and qualities of sensation, are generally considered to be reflected in differences in impulse frequencies in peripheral nerve fibers.

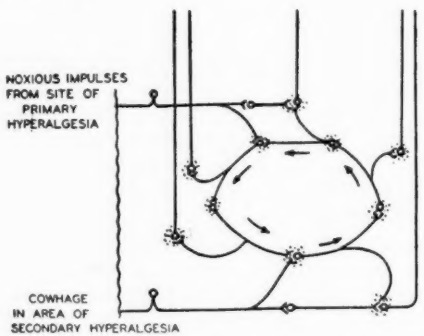

FIG. 6. INHIBITION OF ITCHING IN AREA OF SECONDARY HYPERALGESIA
The stippling represents the excitatory state of internuncial synapses which is responsible for the hyperalgesia and which prevents the formation of circuits necessary for itching.

ITCHY SKIN

Lewis, Grant and Marvin (4) noted that spontaneous itching is an accompaniment of nearly all forms of skin damage, provided that the damage is relatively slight in amount. Bickford (6) further noted that itchy skin surrounds such skin damage. He described "itchy" skin as that which gives rise to an itching sensation when the skin is stroked with the finger, or a blunt object. He extensively investigated and described the phenomenon of "itchy skin" associated with the itch induced by intracutaneous histamine puncture and noted its close relationship to tickle. Goldscheider (12) described the occurrence of itching and "itchy skin" in the palm of the hand accompanying pinching of the web between two fingers. In the following investigations various modifications of both Goldscheider's and Bickford's experiments have been made in an attempt to illuminate further the mechanism of this phenomenon.

Experiment 6. Demonstration of "itchy skin" and hypoalgesia adjacent to a focus of itching

Cowhage was applied to a small (1 cm. in diameter) area of skin on the volar surface of the forearm in various locations. It was found by trial that when it was placed on the wrist near the hand, or somewhat toward the medial aspect of the arm, the phenomenon of "itchy skin" could best be elicited. When the itch had become intense, usually within 30 to 60 seconds after application of the cowhage, stroking of the adjacent skin with the tip of a finger frequently induced an itchy sensation and more frequently intensified the primary itch. This zone extended approximately 2–3 cm. proximally, 1–3 cm. distally and 1–2 cm. laterally. Indeed, when the primary itch had entirely subsided, stroking the surrounding skin often revived it. Pin pricks in this surrounding "itchy skin" zone were dulled, but there was a narrow zone of hyperalgesia entirely surrounding it, where pin prick was experienced as sharper than in control skin. With the passage of time (30 to 60 minutes) after a long-lasting and intense itch, hyperalgesia was noted to have displaced the hypoalgesia and to extend 5 to 10 cm. proximally and 3 to 6 cm. distally. Such hyperalgesia after an intense itch sometimes persisted for several hours.

Experiment 7. Demonstration that the skin about a site of noxious stimulation is "itchy" until the development of secondary hyperalgesia

In 12 experiments on three subjects a small fold of skin on the volar surface of the forearm was painfully pinched by forceps for two to eight minutes. The following description of the observations in an experiment in one subject is characteristic of all of this series (Figure 7). Pin pricks in a 1 cm. wide zone surrounding the pinch during the first 15 seconds intensified the pain of the pinch and there was *hyper*algesia immediately surrounding the pinch. Within the first minute a wider zone of *hypo*algesia to pin prick had developed and extended for 3 cm. proximal and distal to the pinch. In this zone stroking with the finger induced an itchy sensation which was indistinguishable from intense tickle, and itch was readily induced by cowhage. Surrounding this zone there was a narrow band of secondary hyperalgesia in which itch could not be induced either by stroking or by cowhage. In four minutes the hypoalgesic zone extended 3 cm. distally and 5 cm. proximally from the pinch.

It was repeatedly noted that pin pricks within the hypoalgesic zone, although they felt less sharp, accentuated the pain from the pinch. Furthermore, the pain elicited by the forceps gradually diminished. In the fifth, sixth, and seventh minutes when pain was no longer experienced, even rubbing the adjacent area with a finger or a blunt tongue depressor evoked a sensation of itching in the skin as well as at the point of the pinch.

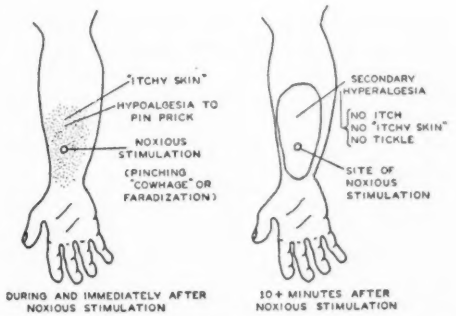

FIG. 7. SENSORY PHENOMENA IN THE SKIN ASSOCIATED WITH NOXIOUS STIMULATION

The pinch was maintained for seven minutes, when the "itchy" area was found to extend 4 cm. distally and 10 cm. proximally from the injury, still surrounded by a narrow band of hyperalgesia. Immediately after removal of the forceps, the hypoalgesic area diminished, its outer borders receding. The hypoalgesia was thus gradually replaced by hyperalgesia. In five to 10 minutes after removal of the forceps, the *hyper*algesia completely filled the zone which had been *hypo*algesic and "itchy." Cowhage applied to this hyperalgesic skin induced no itch, nor was it "itchy" in response to stroking.

The observations in Experiments 6 and 7 of hypoalgesia to pin prick in skin surrounding sites stimulated either by cowhage or by pinching prompted further study of this phenomenon. The experiments of Lewis, and of Hardy, Wolff and Goodell, in which they evoked secondary hyperalgesia by faradic stimulation, were repeated as follows:

Experiment 8. Demonstration of hypoalgesia in skin adjacent to a focus of faradic stimulation

In three subjects a branch of a cutaneous sensory nerve on the volar surface on the forearm was painfully stimulated with faradic current for four minutes. The adjacent skin was tested at the end of one minute and pin prick was found to be dulled. In the fourth minute just before the stimulation ended the pin was appreciated more as pressure than as pain. However, each prick markedly increased the pain from the faradic stimulation. Immediately after the end of the stimulation the hypoalgesic zone extended 4 cm. proximally, 6 cm. distally and 1 cm. laterally from the site of injury. Within 10 minutes no hypoalgesia could be detected. There was now a zone of marked hyperalgesia which extended 8 cm. proximally from the site of faradic stimulation, and 6 cm. distally. No itch or tickle could be elicited by drawing a thread across this area.

Comment

It has been observed when an individual is experiencing one pain that the threshold for pain elsewhere is raised (17). Thus, one possible explanation of the hypoalgesia which was noted in the vicinity of the injured skin in Experiments 7 and 8 might be such an elevation of pain threshold. However, this cannot be the only explanation, because the hypoalgesia was limited in extent and persisted despite decreasing pain intensity as adaptation to the forceps pinch occurred, and for a few minutes after the removal of the forceps. This phenomenon might easily escape attention unless the adjacent skin area were tested during the period of noxious stimulation.

The observations of the last three experiments correspond to those of Experiment 5, in that skin in which secondary hyperalgesia existed was not "itchy" to stroking nor could itching be induced in it by cowhage. Also tickle was not elicited in the zone of secondary hyperalgesia. In short, although itchiness and secondary hyperalgesia may both be associated with skin damage, they do not occur in the same area at the same time.

Experiment 9. Demonstration of the occurrence of both "itchy skin" and itching associated with skin injury

Goldscheider's experiment was repeated in a series of 20 experiments on three subjects. The web between two fingers was tightly pinched with fine forceps. Within half a minute not only could "itchy skin" be demonstrated on stroking of the palm and the back of the hand, but also itch occurred in the palm of the hand spontaneously. Again the "itchy skin" was hypoalgesic to pin prick and was surrounded by a band of hyperalgesia which was *not* "itchy." There was also a small area of hyperalgesia a few millimeters in diameter at the site of the pinch, presumably primary hyperalgesia.

Similarly, pinching a web between the toes for less than two minutes gave rise to spontaneous itching and "itchy skin" in the sole of the foot, especially on the underside of the great toe and the ball of the foot. In one of the subjects, the itching induced by such slight injury between the toes continued for more than an hour.

In one subject itching in and around an insect bite on the medial aspect of the left thigh, about eight inches above the knee, persisted for three days. On the third day a zone of "itchy skin" was found to extend for 5 to 6 cm. around the reddened bite, which was itself spontaneously itching, and hyperalgesic to pin prick. In the zone of "itchy skin" pin prick was dulled, but the area was surrounded by a clearly perceptible narrow band of hyperalgesia. A few pin pricks within this area abolished the itching from the bite for 30 to 60 seconds.

Similar observations were made in a second subject who had "itchy skin" on the top of the left foot associated with a slight abrasion of the skin.

Experiment 10. Demonstration of the spread of itching from a primary focus of itching

In a series of four experiments on two subjects cowhage was placed on an area of skin approximately 1 cm. in diameter on the volar surface of the wrist at the junction between the hand and the arm. Accompanying the intense itch elicited on this site not only was the itchiness in response to stroking of adjacent skin marked, but spontaneous itching which spread into the thenar and hypothenar eminences and into the palm of the hand occurred. Here, again, the area of itchy skin was surrounded by a narrow zone of hyperalgesia which was not "itchy."

Comment

Goldscheider reported that painfully pinching the skin led to the development of hyperalgesia in the surrounding area. He apparently did not notice that the more remote border of hyperalgesia was only a narrow zone and in fact surrounded an area of *hypo*algesia to pin prick. It is only after the passage of several minutes following a pinch of four to seven minutes duration that the hyperalgesia completely displaces the hypoalgesia. Goldscheider inferred that the "itchy skin" was related to hyperalgesia, but from the experiments reported here, it appears that only the skin which is *hypo*algesic to pin prick is "itchy" in response to stroking. These findings are in agreement with the observations that itch is not induced by cowhage in a zone of secondary *hyper*algesia. This is evidence of fundamental similarities between

"spontaneous itching" which follows the application of cowhage or histamine, and that which can be elicited by stroking in skin adjacent to a source of noxious impulses.

Experiment 11. Demonstration of the relation of "itchy skin" and pain, and of the occurrence of "itchy skin" in the absence of touch sensation

In three subjects a blood pressure cuff was wrapped around the upper arm, inflated to 200 mm. Hg, and kept at this pressure for the duration of the experiment. At the end of eight to 15 minutes when pin prick was slightly dulled in the palm of the hand, drawing the end of a tongue depressor across the palmar skin elicited an intense burning itch, and stroking with a finger also elicited tickle or itch. At the end of 20 minutes in all three subjects the sensation of touch in response to laying the flat side of the tongue depressor on the wrist was gone. Itch powder (cowhage) was then applied and within two minutes elicited a burning itch. Stroking the skin both proximal and distal to the cowhage, was felt as "itchy" and also intensified the itch from the cowhage. The zone in which "itchy" skin could be detected was well demarcated for a distance of 8–10 cm. proximal to the itch spot, and throughout the palm to the tips of the fingers. In the distal zone of "itchy skin" no touch sensation could be detected.

Comment

Bickford reported that the perception of "itchy skin" failed at a stage of asphyxia at which both touch and pain sensibility were still present, and was therefore forced to postulate that special peripheral nerves transmitted the sensation. The observations described above, however, are evidence that "itchy skin" can occur in the absence of touch as long as pain is preserved.

They indicated that both itch and "itchy skin" occur independently of touch sensation, and as long as some fibers are still conducting pain impulses. Perhaps Bickford failed to make this observation because he used intracutaneous histamine puncture to produce spontaneous itching and "itchy skin" and in our experience the latter was less intense and more difficult to detect when histamine was used to evoke itch.

Discussion

The "itchy skin" phenomenon is indistinguishable subjectively from tickle (see next section). The sensation evoked by light stroking of otherwise unstimulated skin is called "tickle," that evoked by light stroking of skin in the neighborhood of a source of itching has been called "itchy," but they are otherwise alike, and both provoke a desire to scratch. "Itchy skin" is like itch and tickle in that it could not be elicited in skin manifesting secondary hyperalgesia. Bickford observed that if it was impossible to elicit "itchy skin" as a result of nervous system disease or of experimental procedures, it was also impossible to elicit tickle. He concluded that the development of "itchy skin" depended on axon-reflexes similar to those responsible for the development of the flare around sites of skin injury. Whether or not this is correct, the "itchy" sensation elicited in the area presumably results from a kind of activity in internuncial neurons similar to that which is responsible for the primary itching at the site of cowhage application.

TICKLE

When a stiff nylon thread is drawn across the skin a peculiar esthetic experience usually ensues, which long outlasts the period of stimulation. This is best demonstrated on the margin of the upper lip. The sensation has two distinct components. The first of these resembles itch and has in common with it an associated urge to rub or scratch the skin. The second consists of an awareness of movement of a light object touching the skin. In some parts of the body, *e.g.*, the finger pads and the extensor surface of the elbow, only the second component is prominent, whereas at the lip margin the itching component is conspicuous. It is for the itching component of the sensation evolved by a moving stimulus that the term "tickle" has been reserved in this paper.

In the following experiments the relation of tickle, itch, "itchy skin" and pain were further investigated.

Experiment 12. Demonstration that tickle and itch differ only in that tickle has the additional element of movement

A fine nylon thread was touched to the skin of the forehead in one spot. Ten subjects so tested reported that the sensation elicited was itch, and was accompanied by the urge to rub or scratch. When the thread was lightly *drawn across* the skin, some of the subjects changed their report of the sensation to tickle, but stated that the only change in the quality of the experience was the additional perception of movement of the thread, although the desire to rub or scratch was intensified.

In six subjects, the difference between touch and tickle or itch was clearly demonstrated by touching first the pad of one finger and then the forehead. A light touch by the finger of the experimenter on the finger pad of the subject elicited only touch, but on the forehead, itch or tickle were elicited, with an invariable accompaniment of a desire to rub or scratch the stimulated skin.

Comment

These observations make clear that the conception of tickle in everyday experience conforms exactly to the definition given above, *i.e.*, tickle is the itching component of the sensation evoked by a light moving stimulus on the skin.

Experiment 13. Demonstration that tickle is obliterated by stimulation of pain endings

The forehead was rubbed briskly so that a slight after-pain was experienced, and again tested by lightly drawing the nylon thread across the skin. Now the sensation elicited was purely that of touch without the unpleasant component arousing the urge to rub or scratch. Similarly, immediately after the forehead was pricked vigorously with a pin, for at least as long as the faint after-pain of the pricking persisted the sensation elicited by the thread was again only touch.

Comment

Although drawing a wisp of cotton across the skin in most skin areas is used as a bedside testing procedure to indicate perception of light touch, it is also used on the upper lip, a very "ticklish" area, to test for the integrity of fiber systems conveying noxious impulses in the skin supplied by the fifth cranial nerve.

It is apparent that the drawing of a thread or wisp of cotton across the skin may under varying circumstances give rise to qualitatively distinct sensations. As stated above, it has seemed profitable to reserve the word "tickle" for that part of the sensation thus elicited which resembles itch and arouses the desire to rub or scratch. The effect of painful stimuli in abolishing or preventing tickle is apparently analogous to the phenomenon of abolishing itch by pin prick.

Experiment 14. Demonstration of the absence of tickle in a zone of secondary hyperalgesia

In two subjects zones of secondary hyperalgesia were defined on the volar surfaces of the forearms after faradic stimulation of a superficial cutaneous sensory nerve. When the hyperalgesia was fully developed a nylon thread drawn lightly across the area elicited a sensation of touch; when the thread was applied with slightly greater force, touch commingled with pain was elicited, but the sensation was definitely not tickle. The same thread elicited tickle in control areas on the opposite arm, and in areas of skin on the same arm outside of the area of secondary hyperalgesia. In these two subjects the secondary hyperalgesia was abolished by pin prick within its borders. Immediately thereafter, tickle could again be elicited.

Comment

It appears that tickle, like itch, is inhibited by the presence of secondary hyperalgesia of the skin; that a stimulus clearly eliciting tickle in control skin, elicits in hyperalgesic skin either a sensation of touch without the special quality of tickle or itch, or of touch commingled with pain. Areas of secondary hyperalgesia have thus been demonstrated to be "anti-ticklish" as well as "anti-pruritic."

Experiment 15. Demonstration of the occurrence of tickle in the absence of touch sensation

In three subjects a blood pressure cuff was inflated about the upper arm at a pressure of 200 mm. Hg. About 20 minutes later the light touch of a flat tongue depressor could not be perceived on the back and side of the forefinger. At this time, however, a stiff nylon thread drawn across this area elicited a distinct tickle which merged into an itching sensation

Comment

It has been previously noted that during asphyxia of an arm touch sensation disappears at a time when pain can still be elicited. This experiment demonstrates that tickle, like itch, is independent of touch, and is indeed also probably carried over the afferent fibers which mediate pain.

Discussion

No general agreement on the relation of the sensation of tickle to touch, pain and itch has previously been reached. Discussion has focussed on whether tickle was essentially different from itch, and whether the mediation of tickle sensation was to be assigned to touch or to pain receptors.

With regard to the first question, Murray (21) reported that her subjects found that itch differed from tickle only in being "more persistent, painful and intolerable." Torök (22), on the other hand, asked patients with pruritus to compare their spontaneous itching with the sensation elicited by drawing a wisp of cotton across the skin. From these comparisons he concluded that itch and tickle

were different sensations which could be distinguished by his subject. It should be noted, however, that motion adds an element to the sensation which makes it different from itching, and probably accounts for the patients' statements that they could distinguish between the two. It is also important that under certain circumstances, as discussed above, the same moving stimulus provokes a sensation which induces no desire to scratch, consists entirely of touch, and is not ordinarily called tickle by the subject experiencing it. It is conceivable that difficulties of communication with patients might lead to a report of "tickle" for this sensation also.

Thöle (11) also separated itch and tickle as regards the structures involved, concluding that tickle was related to stimulation of touch receptors. He failed to make the observation that tickle can be elicited in areas of skin in which touch is no longer present, nor did he differentiate between true tickle and a sensation of moving touch in areas where pain could not be elicited.

Bishop (23, 24) suggested that tickle sensation was a function of touch rather than pain receptors. He found that weak and rapid electrical stimulation of touch endings failed completely to elicit tickle but this he attributed to the lack of movement of the stimulus. He argued that tickle can be elicited by a contact with the skin so light that it is clear that the receptors concerned must have a very low threshold, and that therefore participation of pain receptors is out of the question. In his own observations on cutaneous sensation, however, he found that in most areas of skin, except notably on the balls of the finger, prick has a *lower* threshold than touch. Furthermore, in reviewing (25) von Frey's observations, Bishop calls attention to the fact that in using mechanical stimulators of small diameter, thresholds for touch and pain closely approximate each other. Likewise, Hardy, Wolff, and Goodell (10), testing for touch threshold with von Frey hairs in hyperalgesic and control areas of skin, found that the thresholds for touch and for pain were not grossly different. Bishop's second piece of evidence linking tickle with touch was that tickle, like touch, "adapts" to continued stimulation (24). However, continuous noxious stimulation also ceases to produce pain, as can be readily demonstrated by holding a pin point steadily against the skin for several seconds, or by pinching a fold of skin with forceps.

Bickford observed that tickle, "itchy skin," itch, and pain were all absent in patients with anterolateral tract lesions, even though touch sensation remained intact.

Pritchard (26) considered that tickle, itch and pain all lay on the same continuum of sensation, and that tickle, like itch, was a variety of pain. He nevertheless concluded that tickle and pain involved different peripheral pathways. He failed to present evidence that tickle could be elicited in the complete absence of pain. On the other hand, Sarnoff and Arrowood (27) reported that tickle sensation elicited "by scratching the soles of the feet" persisted in some of their patients in the absence of pain from pin prick during spinal block with procaine. However, the intensity of their scratch stimulus was not described, and it is not clear that it elicited true tickle, especially since there was no description of the qualities of the sensation experienced. It is also possible that their observations may be related to Thöle's findings (11).

Zotterman (28) on the basis of his studies of axone potentials in cat nerves concluded that tickle and itching sensations are mediated by the fibers which are responsible for the pain elicited by pin prick. He also observed that tickle could not be elicited in patients with analgesia of the face following trigeminal tractotomy, although touch sensibility was intact.

On the basis of the observations reported here it is inferred that the sensation called "tickle" is mediated by the same neural fibers as are involved in itch and pain. This conclusion is based on the experiments involving differential anesthetization, for tickle could be elicited when touch sensitivity was absent and pain sensitivity retained, thus exactly paralleling cowhage-induced itching in like circumstances. Furthermore, tickle is inhibited in an area of secondary hyperalgesia, as is itch; and it is obliterated in skin to which a painful pricking or rubbing has been applied, just as itch is obliterated by pricking in adjacent areas of skin. It is concluded, therefore, that the pathways concerned in the perception of tickle are those for pain, and that touch mechanisms do not participate, except to add something which is essentially extraneous.

SUMMARY AND CONCLUSIONS

1. The sensation of itch has two subjectively distinguishable components, one pricking and the other burning. These correspond to the two kinds of cutaneous pain, and are mediated respectively by the two types of nerve fibers involved in the transmission of pain from the skin. Touch receptors and fibers are not involved.

2. Cutaneous tickle and the sensation elicited in "itchy skin" do not differ qualitatively from itch, except by the addition of an awareness of movement, and are mediated by the same neural structures, *i.e.*, those which transmit pain. Touch receptors, when functioning, probably add an essentially extraneous component to tickle and to the sensation elicited in "itchy skin," but both phenomena occur in the absence of touch.

3. When itching is present, the pain threshold at the site is lower than it is in the same skin during itch-free intervals.

4. Itch occurs when pain receptors are weakly stimulated.

5. Tickle, itchy skin and itch are abolished by pin pricks in adjacent skin. In the case of itch, this abolition is possible if the skin is pricked anywhere in the dermatome which contains the site of itching.

6. Tickle, "itchy skin," and itch do not occur in areas of secondary hyperalgesia. They do occur in skin surrounding sites of noxious stimulation when the area is hypoalgesic to pin prick.

7. It is suggested that the sensation of itch results from the presence in the spinal cord of impulses traveling in circuits of internuncial neurons, with a consequent patterned discharge up the spinothalamic tracts. Such circuits are presumably established when peripheral pain nerves discharge into the cord at a low frequency. When the circuits are broken up—by strong stimulation in the same dermatome, for instance—itching ceases.

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THE EFFECT OF ORAL ADMINISTRATION OF CALCIUM FRUCTOSE DIPHOSPHATE ON THE SERUM ORGANIC PHOSPHATE, INORGANIC PHOSPHATE, CALCIUM, PROTEIN, AND CITRIC ACID LEVELS[1]

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It has been clearly demonstrated that the rate of glucose absorption is independent of the concentration of the glucose solution introduced into the intestine (1–4). This has been interpreted as indicating that glucose absorption is an enzymatic process in which phosphorylation at the wall of the intestine precedes the absorption of glucose (5–7). This mechanism has been thoroughly tested and evidence in its favor is convincing (8–10). It appeared to us, therefore, that hexosephosphates introduced into the intestinal tract might be absorbed directly, without splitting of the phosphate linkage with the exception of that material which would be hydrolyzed by stomach acids and destroyed by the intestinal flora. It was therefore decided to determine the manner in which a hexosephosphate given orally to adults is absorbed. The adults studied were patients taken from our wards who showed no evidence of impaired intestinal absorption.

The administration of large amounts of inorganic phosphate is not an innocuous procedure in that one runs the risk of reducing the calcium ion concentration to a low level with resultant tetany. It was therefore decided, in this preliminary study, to administer calcium simultaneously. This could conveniently be done by administering the hexosephosphate in the form of its calcium salt. Calcium fructose diphosphate is readily available and was, therefore, the substance chosen.

The calcium fructose diphosphate was prepared by the Ernst-Bischoff Company,[3] in the form of a fine powder, and also in pills containing 0.5 gram of this material. If we assume that one molecule of this material contains two atoms of calcium and two phosphate groups, then the empirical formula is $C_6H_{12}O_{12}P_2Ca_2$ and the molecular weight, 418. Theoretically, 14.8 per cent phosphorus and 19.1 per cent of calcium should be present in the molecule. On analyzing the product, 13.6 per cent of phosphorus was obtained and 17.2 per cent of calcium.[3]

Calcium fructose diphosphate is apparently readily hydrolyzed. It was observed that preparations which had been kept in stoppered bottles, but which had been opened frequently over a nine months' period, lost a high percentage of their organic phosphate content, the phosphate in the material becoming inorganic. The material used, therefore, was tested for its inorganic phosphate content. If the material contained more than 2 per cent of its phosphate in the inorganic form, it was discarded. Calcium fructose diphosphate is more soluble than calcium phosphate, and a preliminary test may be made by shaking up 20 mg. in 25 ml. of water. The material should dissolve readily with no residue.

The criteria of whether the hexosephosphate has reached the blood stream were determined by the organic phosphate content of the serum after the proteins had been removed. Inorganic phosphate was also measured. Calcium and two substances which normally form complexes with calcium, namely, citric acid and protein, were also estimated. Calcium was done by the method of Sobel and Sobel (11). Inorganic phosphorus was done by a modified method of Fiske and Subbarow (12, p. 579). Total protein was done by a modified biuret method (13). Citric acid was done by the method of Natelson, Pincus, and Lugovoy (14). Determination of total non-protein phosphorus was done by digestion of the trichloracetic acid filtrate of cell free serum (12, p. 581). When 50 μg. of phosphorus as calcium fructose diphosphate or as calcium glyceryl monophosphate were added per ml. of serum, 94 to 102 per cent of the phosphorus added was recovered by this method.

As determined by recoveries of added amounts of various constituents to serum, citric acid was determined with an accuracy of 5 per cent, inorganic phosphorus with an accuracy of 3 per cent and calcium with an accuracy of 3 per cent. Per ml. of serum, 20 μg. of citric acid, 40 μg. of inorganic phosphorus, and 50 μg. of calcium were added

[1] Presented at the Federation Meeting of the American Societies for Experimental Biology at Atlantic City, April, 1950.

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[3] Calcium fructose diphosphate is manufactured by the Ernst Bischoff Co. of Ivorytown, Conn., under the trade name of Com-B-Phos. The material supplied us contained some water of crystallization. The company makes an anhydrous product which they claim is more stable.

TABLE I

Change in serum levels after administration of 6 grams of calcium fructose diphosphate

Case* no.	Time	Non-protein phosphorus (*mg.* %)			Calcium	Citrate	Protein
		Total	Inorganic	Organic			
	hrs.				*mg.* %	*mg.* %	%
1	Fasting	3.9	3.3	0.6	10.1	2.03	7.3
	1	5.8	3.2	2.6	10.3	1.91	7.5
	2	6.7	3.3	3.4	10.5	2.03	7.3
	3	6.1	3.5	2.6	10.4	2.00	—
2	Fasting	3.1	2.7	0.4	9.7	1.89	6.3
	1	5.6	2.9	2.7	10.3	2.20	6.4
	2	6.1	3.4	2.7	10.2	1.93	6.2
	3	4.7	3.0	1.7	10.3	2.03	6.5
3	Fasting	3.4	3.0	0.4	9.0	2.34	7.2
	1	4.4	3.7	0.7	9.3	2.56	7.3
	2	5.8	4.1	1.7	9.6	2.33	7.5
	3	6.7	3.8	2.9	9.6	2.28	7.3
4	Fasting	3.5	2.9	0.6	8.3	2.44	5.6
	1	4.4	3.1	1.3	8.5	2.44	5.7
	2	7.5	3.1	4.4	8.8	2.71	5.8
	3	5.3	3.1	2.2	8.3	2.49	5.6
5	Fasting	4.7	3.5	1.2	10.3	2.25	5.9
	1	5.8	3.7	2.1	10.5	2.70	5.9
	2	7.0	4.3	2.7	10.6	2.05	6.1
	3	5.8	4.3	1.5	10.6	2.20	6.1
6	Fasting	5.6	4.5	1.1	9.5	1.95	6.9
	1	7.0	5.0	2.0	10.3	2.40	6.9
	2	8.1	5.0	3.1	10.3	1.95	7.0
	3	7.0	5.0	2.0	10.3	1.95	7.2
7	Fasting	4.9	4.2	0.7	10.1	2.36	7.1
	1	5.7	4.4	1.3	11.0	2.42	7.3
	2	5.6	4.5	1.1	10.9	2.61	7.2
	3	5.3	4.6	0.7	10.8	2.29	7.3
8	Fasting	3.9	3.4	0.5	10.2	1.84	6.5
	1	5.0	3.6	1.4	10.9	2.06	6.6
	2	5.1	3.7	1.4	10.8	2.06	6.6
	3	4.6	3.5	0.9	10.8	2.03	6.5
9	Fasting	3.7	2.6	1.1	9.9	2.36	6.7
	1	4.2	2.8	1.4	10.2	2.51	6.7
	2	4.7	2.9	1.8	10.2	2.36	6.8
	3	5.2	3.0	2.2	10.4	2.30	6.9
10	Fasting	4.6	3.4	1.2	10.2	2.18	6.8
	1	5.0	3.4	1.6	10.2	2.30	6.7
	2	6.9	4.0	2.9	10.4	2.18	6.8
	3	6.5	3.9	2.6	10.5	2.14	6.8
11	Fasting	3.7	3.5	0.2	9.8	1.60	7.2
	1	4.2	3.8	0.4	9.8	1.70	7.6
	2	4.2	3.8	0.4	10.3	1.86	7.4
	3	4.4	4.1	0.3	9.4	1.43	7.4
12	Fasting	4.2	3.9	0.3	8.1	2.10	7.5
	1	5.0	4.5	0.5	8.5	2.31	7.7
	2	5.5	4.8	0.7	8.0	2.14	7.4
	3	5.3	4.8	0.5	8.3	1.90	7.4

* Hospital patients with apparently no impairment of intestinal function. Results are averages of duplicates.

for the recovery determinations. The accuracy of the protein method was estimated at approximately 3 per cent by comparing the procedure used with the Kjeldahl procedure on 50 samples, in the routine laboratory. All analyses were done in duplicate and the tables below show the results of the average of these duplicates.

The experiments were performed in the following manner: A fasting sample of blood was drawn and 6 grams (12 pills), of calcium fructose diphosphate were taken by mouth. Thereafter, samples of blood were taken at intervals of one hour for three hours.

Results are shown in 12 cases in Table I (cases 1 through 12). Summary of these results is shown in Tables II and III where the various determinations are averaged for cases 1 through 12. Table IV shows the results carried out with a calcium fructose diphosphate powder preparation which had been hydrolyzed by nine months' exposure to moisture in the powder and air. On analysis, approximately 80 per cent of the phosphorus was in inorganic form. These results serve as a "blank" for comparison of the absorption of a hexosephosphate with a phosphate preparation which contained the same quantity of calcium and phosphorus, but little organic phosphate.

DISCUSSION

Examination of Table III shows a rise of approximately 15 per cent in inorganic phosphorus, and a rise of 173 per cent of the non-protein organic phosphorus, over the fasting level. That these results are statistically significant is apparent from Table I where increases are observed in every case without exception. The control table (Table IV) indicates that the total rise in organic phosphorus levels could not be caused by the ingestion of inorganic phosphate. In view of the small amount of organic phosphate normally present in cell-free serum, the 173 per cent rise is somewhat misleading. An estimation of the amount of organic phosphate absorbed is best obtained by examination of the average absolute rise in non-protein organic phosphate. This is listed as 1.40 mg. per 100 ml. in Table III. Assuming that 20 per cent of the body weight represents total extracellular fluid, the average adult would have 20 per cent $\times$ 75 Kg. = 15 liters of extracellular fluid. The total non-protein organic phosphorus increase would then be $1.4 \times 10 \times 15 \div 1000 =$ 0.210 gram. Since the phosphorus represents only 13 per cent of the calcium fructose diphosphate

TABLE II

Average values for the 12 cases studied
(Table I, cases 1–12)

	Calcium			Total protein			Citric acid		
	Mg. %	Mg. % rise over fasting	% Rise over fasting	Mg. %	Mg. % rise over fasting	% Rise over fasting	Mg. %	Mg. % rise over fasting	% Rise over fasting
Fasting	9.59	—	—	6.76	—	—	2.06	—	—
1 hr.	9.90	0.31	3.2	6.80	0.04	0.6	2.28	0.22	10.7
2 hrs.	10.01	0.42	4.4	6.80	0.04	0.6	2.19	0.13	6.8
3 hrs.	9.95	0.36	3.8	6.73	0.03	0.4	2.10	0.04	1.9

used, this would imply that 1.6 grams of fructose diphosphate is present in the extracellular fluid at the time of the peak rise in nonprotein organic phosphorus as an average for the 12 adults. The one hour, two hour and three hour figures in Table III for organic phosphorus indicate that a continuous process of absorption and metabolism is going on. From this observation and the fact that some fructose diphosphate is probably hydrolyzed by the acid in the stomach, one might infer that a high percentage of the fructose diphosphate which reaches the intestine is rapidly absorbed. The speed of absorption is indicated by the fact that a rise in non-protein organic phosphorus can be demonstrated in one hour and that a peak is reached in two hours. This compares in speed with the absorption of glucose. A more accurate picture would have been obtained if enteric coated pills had been used.

It is of interest that at no time did the inorganic phosphorus levels rise to a point where a hazard of tetany might be encountered. At the peak, the inorganic phosphorus rise was less than one-third that of the rise in organic non-protein phosphorus. This rise in inorganic phosphorus is equivalent to 0.59 gram of calcium fructose diphosphate. Thus at the peak rise, more than one-third of the substance administered (6 grams) could be accounted for in the extracellular fluid (1.6 grams + 0.59 gram = 2.19 grams), calculated on the rise in total phosphorus.

The ratio of the phosphorus to calcium absorbed should have been 31/40 or approximately 3/4 if the calcium fructose diphosphate molecule had been absorbed directly. Actually the ratio was 1.91/0.42 (see Tables III and IV), or 9/2 approximately. Disproportion between the absorption of calcium and organic phosphorus may be interpreted on the basis that the molecule is not absorbed as a whole, but that in the intestinal tract calcium dissociates, and the fructose diphosphate is absorbed in greater amounts than calcium. This could only be demonstrated if the amount of calcium excreted in the stool were measured. In the present study stool analyses were not done.

The relatively slight increase in calcium concentration (Table II) suggested that a large portion of the calcium may be tightly bound in organic form. However, ashing made no significant difference (2 per cent increase) in the calcium content of the same serum at the peak organic phosphorus level. Increase in citrate levels is

TABLE III

Average values for the 12 cases studied
(Table I, cases 1–12)

	Total non-protein phosphorus			Inorganic phosphorus			Organic non-protein phosphorus		
	Mg. %	Mg. % rise over fasting	% Rise over fasting	Mg. %	Mg. % rise over fasting	% Rise over fasting	Mg. %	Mg. % rise over fasting	% Rise over fasting
Fasting	4.19	—	—	3.40	—	—	0.79	—	—
1 hr.	5.17	0.98	23	3.66	0.26	7.65	1.51	0.72	92
2 hrs.	6.10	1.91	46	3.91	0.51	15.00	2.19	1.40	173
3 hrs.	5.58	1.39	33	3.88	0.48	14.10	1.70	0.91	116

TABLE IV

Change in serum levels after administration of 6 grams of hydrolyzed calcium fructose diphosphate

Case* no.	Time	Non-protein phosphorus (*mg.* %)			Calcium	Citrate	Protein
		Total	Inorganic	Organic			
	hrs.				*mg.* %	*mg.* %	%
13	Fasting	4.2	3.9	0.3	9.0	1.90	6.0
	1	4.6	4.1	0.5	9.6	2.20	6.0
	2	4.6	4.1	0.5	9.5	2.10	6.2
	3	4.6	4.2	0.4	9.4	1.90	6.0
14	Fasting	4.4	3.8	0.6	10.0	2.20	7.4
	1	5.0	4.3	0.7	10.0	2.10	7.5
	2	5.3	4.5	0.8	10.3	2.33	7.2
	3	5.5	4.8	0.7	10.1	2.25	7.2
15	Fasting	3.5	3.0	0.5	10.0	2.20	7.3
	1	3.9	3.3	0.6	10.3	2.36	7.6
	2	4.3	3.5	0.8	10.5	2.33	7.6
	3	4.8	4.0	0.8	10.2	2.33	7.3
16	Fasting	8.2	7.6	0.6	8.0	2.10	5.0
	1	8.2	7.4	0.8	8.7	2.54	5.4
	2	8.1	7.4	0.7	8.7	2.15	5.0
	3	8.2	7.5	0.7	8.1	1.92	5.0
17	Fasting	6.2	5.5	0.7	7.7	1.92	4.7
	1	6.0	5.5	0.5	8.1	2.03	4.8
	2	6.0	5.4	0.6	7.6	2.06	4.7
	3	6.2	5.4	0.8	7.6	1.96	4.7
Average	Fasting	5.3	4.8	0.5	8.9	2.06	6.1
	1	5.4	4.9	0.5	9.3	2.25	6.2
	2	5.6	5.0	0.6	9.3	2.20	6.1
	3	5.9	5.2	0.7	9.1	2.06	6.0

* Hospital patients with apparently no impairment of intestinal function. Results are averages of duplicates.

probably associated with the rise in calcium levels (15). Protein changes were not considered significant.

Certain pathological conditions exist such as hyperparathyroidism (16), and low phosphorus rickets (17–19), where the phosphate levels of the blood cannot be maintained even when inorganic phosphate is administered. In lead poisoning, the administration of phosphorus has been suggested in order to alleviate symptoms by removal of lead from the plasma (20–22). This technique has been hampered by the danger associated with injections of inorganic phosphorus. The authors intend to investigate the possibility that hexose-phosphates may find application in these cases.

The properties ascribed to calcium fructose diphosphate in this report are apparently not limited to fructose diphosphate since calcium glycerol phosphate, when tested in one case, produced similar results. It is the intention of the authors to extend this study to other phosphate esters such as glucose 6 phosphate, and glucose 1 phosphate.

SUMMARY

1. The effect of 6 grams of calcium fructose diphosphate administered orally on the calcium, protein, citric acid, inorganic phosphorus and non-protein organic phosphorus levels of the serum was studied in 12 adults.

2. The results show an average increase in the calcium concentration of 4.4 per cent. Citric acid rose 10.7 per cent over the fasting levels. Inorganic phosphorus levels rose an average of 15 per cent. The greatest change was noted in the case of non-protein organic phosphorus which was of the order of 173 per cent increase.

3. Six grams of hydrolyzed calcium fructose diphosphate given orally did not cause a similar rise in non-protein organic phosphate in five cases.

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THE CLINICAL DETERMINATION OF PROTEIN-BOUND IODINE[1]

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The value of plasma protein-bound iodine (PBI) measurement in the evaluation of thyroid disease has been well established (1–7), but it has not yet assumed its deserved place in the clinical armamentarium because of the technical difficulties involved in distillation procedures. Modification of an alkaline-incineration technique (8) has resulted in the evolution of a method for PBI determination which is practicable for a clinical laboratory. It must be appreciated that extreme care will always be required in the quantitative measurement of 0.05 μgm. PBI/cc. plasma as well as in avoiding contamination.

SOLUTIONS REQUIRED

All solutions should be made up in double-distilled water. If the amount of iodide in the water is still too high to give a satisfactory blank, it may be necessary to carry out these distillations with such reagents as alkali and permanganate.

All reagents used should be of highest purity obtainable. However, the iodine content in each instance must be judged from the blank values rather than from estimates shown on the labels. Often it is necessary to turn to a new source of supply or a different lot number in case of contamination.

Zinc sulfate. 100 grams of $ZnSO_4 \cdot 7\ H_2O$ per liter of solution.

Sodium hydroxide. 0.5 N is 20 grams of NaOH per liter of solution. The zinc sulfate and sodium hydroxide reagents should be so related that when 10.0 cc. of the former are diluted with 50–70 cc. of water and titrated with the latter, 10.8–11.2 cc. of the 0.5 N NaOH are required to produce a faint permanent pink color with phenolphthalein.

Hydrochloric acid, 2 N. 200 cc. C.P. concentrated HCl per liter of solution.

Sulfuric acid, 7 N. 196 cc. C.P. concentrated H_2SO_4 per liter of solution.

Iodide standards. Either NaI or KI of highest purity desiccator-dried may be used. If NaI is chosen, 118.1 mg. are dissolved in water and made to 1 l., if KI, 130.8 mg. These concentrated stock solutions contain 100.0 μgm. I/cc.

A more dilute stock is obtained by diluting 2 cc. of the above solution to 1 l. To prepare the standard of 0.04 μgm. I/cc. solution used in each determination, 10 cc. of this dilute stock are further diluted to 50 cc.

All of these solutions have been found to keep indefinitely at refrigerator temperatures, but it is preferable to make up the most dilute standard fresh every week or two.

Ceric ammonium sulfate, 0.02 N. 12.65 grams of the salt are dissolved in 500 cc. of water plus 230 cc. of 7 N H_2SO_4. When the solution is clear, it is made to 1 l. with water.

Sodium arsenite, 0.1 N. Dissolve 4.95 grams of As_2O_3 in 25 cc. of 4% (1N) NaOH, warming to hasten solution. This should be diluted with about 300 cc. of water and dilute sulfuric acid added until the solution is slightly acid to litmus paper (if the 7 N is used, about 4 cc. will be required). The solution is then made to 1 l. If sodium arsenite itself is used, 6.50 grams of $NaAsO_2$ should be dissolved in distilled water and diluted to 1.0 l. No acid or alkali is required with this salt.

ANALYTICAL PROCEDURE

The steps involved can most conveniently be described under four headings: *1*) precipitation and washing of plasma proteins, *2*) drying and incineration of sample, *3*) dissolving iodide from the ash, *4*) colorimetric determination of iodide present.

1) Precipitation (9) *and washing of plasma proteins.* The blood sample, about 10 cc., is centrifuged and the plasma or serum drawn off. Duplicate aliquots of 1 cc. each are pipetted into 15 × 125 mm. Pyrex test tubes, diluted with 7 cc. of distilled water and 1.0 cc. of 10% zinc sulfate solution added. The contents of each tube are mixed with a glass stirring rod about 2 mm. in diameter, 1.0 cc. of 0.5 N sodium hydroxide is added and the solutions thoroughly mixed to insure even distribution of the alkali and complete precipitation of the proteins. Any material adhering to the rod can be removed by rubbing it with a rotary movement against the inside wall of the tube.

The tube and contents are centrifuged for 10 minutes to pack the precipitate and the supernatant fluid is poured off. Ten cc. of distilled water are added and the protein resuspended by means of the same stirring rod

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[2] Deceased June 21, 1949.

originally used. Stirring should be only sufficiently vigorous to distribute the zinc proteinate uniformly throughout the solution; if over-zealously carried out, the precipitate may be so finely divided that it cannot easily be centrifuged down. Centrifugation is again carried out and the supernatant discarded. This washing process is repeated two more times, making a total of three washings (the original precipitation is not included as a washing).

2) Drying and incineration. After the last supernatant solution has been poured off, 0.8 cc. of 4.0 N sodium carbonate is added and thoroughly stirred into the precipitate with the same stirring rod used in the washings. Removal of material clinging to the rod can be accomplished by rubbing as described previously, followed by 0.2 cc. of the 4.0 N Na_2CO_3 added dropwise down the rod. This last portion of alkali can easily be mixed with the sludge already present without use of the stirring rod. The tube is then placed in an oven set at 85–95° C. to drive off water. This usually requires 12–18 hours and is usually carried out overnight.

After thorough drying of the alkali and zinc proteinate, ashing is carried out by placing the tube in a muffle furnace for two and one half hours at 600 ± 25° C. At the end of that time, the tube is removed and allowed to cool to room temperature.

3) Dissolving iodide from the ash. Two cc. of 2 N hydrochloric acid are added with due caution to avoid excessive effervescence. A glass rod is used to mix any unreacted ash with the acid, and 2.0 cc. of 7 N sulfuric acid and 3.0 cc. of water are added. The contents of the tube are stirred until the reaction appears to be finished, transferred to a clean tube and briefly centrifuged to pack the insoluble material.

4) Determination of iodide (10). For each tube a pair of colorimeter tubes is taken. To one of the pair is added 1.0 cc. of distilled water containing 0.04 μgm. of iodine as sodium or potassium iodide and to the other is added 1.0 cc. of water. A 3.0 cc. aliquot of the supernatant in the sample tube is pipetted into each colorimeter tube and 0.5 cc. of the sodium arsenite solution is added to each tube. Mixing should be carried out by some technique which avoids both loss of solution and contamination.

The colorimeter tubes are then placed in a well-stirred constant temperature water bath maintained at 39 ± 0.1° C. for 10 minutes to come to temperature. The ceric ammonium sulfate solution should also be warmed in the bath. Since the actual determination of iodide is accomplished by measuring iodide catalysis of the rate of decolorization of yellow ceric ammonium sulfate by arsenious acid, the times at which colorimeter readings are made must be definitely set up. One cc. of the ceric solution must be added to each tube with adequate time allowance for reading the color change in the previous tube. This is accomplished in this laboratory by leaving a 30-second interval between tubes and by setting up a maximum of only 12 tubes in a single series. Colorimeter readings are then made at six and 12 minutes after adding the ceric sulfate solution to each tube. If 30 seconds is not adequate for making the reading, more time must be allowed, and fewer determinations done in any one series. Since the measurement depends entirely upon the decrease in amount of color, the pipetting of the 1.0 cc. of ceric solution must be highly accurate, even though done rapidly. Mixing must again be rapid and thorough.

The Klett-Summerson readings (using blue filter No. 42) are plotted on semi-log paper against the time in minutes. Ordinary graph paper is suitable for instruments giving readings in terms of per cent light transmission. The colorimeter setting at zero time is obtained for any given batch of reagents by quickly reading a reagent blank immediately after addition of the ceric sulfate. The lines describing the two reaction rates are drawn using the points for 0, 6 and 12 minutes, as is shown in Figure 1 and then the horizontal line indicating times required to reach equal amounts of decolorization. The amount of iodide in the colorimeter tube without added iodide is calculated from the following equation, obtained from similar triangles (see Figure 1):

$$\mu\text{gm. I in colorimeter tube} = \frac{0.04 \times t_2}{t_1 - t_2}$$

t_1 = time in minutes at intercept of the rate of decolorization in tube containing no extra iodide with the designated horizontal.

t_2 = time in minutes at intercept of the rate of decolorization in tube containing added iodide with the designated horizontal.

The reagents used contain variable amounts of iodide and the total must be determined by blank analyses starting with the zinc sulfate and sodium hydroxide solutions and carried through the entire procedure, including the washings with 10 cc. of distilled water, addition of 1 cc. of sodium carbonate, drying, ashing, treatment with hydrochloric and sulfuric acids and the final colorimetric assay on 3 cc. aliquots just as with an unknown sample. The blank value calculated by the same formula shown previously has been 0.015 − 0.017 μgm. I/tube in this laboratory.

Since it is customary to express the values in terms of μgm./100 cc. plasma, the complete calculation would follow:

$$\text{Plasma PBI } \mu\text{gm. I/100 cc.} = 100 \times \frac{7}{3} \text{ (I in final aliquot—blank I)}$$

where the factor of 7/3 has been introduced to correct for the 3.0 cc. aliquot taken for colorimetric evaluation from the 7.0 cc. of acid supernatant from the ashed sample.

If so much iodide is present in the sample that decolorization is nearly complete in the second tube, with 0.04 μgm. of added I, the determination may be repeated using less than the 1.0 cc. of plasma usually called for. Computation of the results must be adjusted as necessary to cover this change.

NOTES AND PRECAUTIONS

It must be emphasized that the various conditions explicitly detailed herein have been evolved after intensive study. Modifications to suit any

other laboratory are quite in order, but they must be rigorously tested before being incorporated into the procedure.

General. Scrupulous care must be exercised to insure clean glassware. A detergent-soap mixture such as "Lakeseal" [3] is preferred for routine cleaning. This solution is rinsed off with tap water followed by double-distilled water. Contamination, especially from iodine and mercury, must be guarded against. Since elemental iodine is so volatile and in such prevalent use as an antiseptic, this comprises the greatest hazard. Tincture of iodine must be avoided as an antiseptic on the skin of the patient before the sample is drawn and of any persons carrying out the actual determination. If elemental iodine is being used in any chemical determination or histological procedure in the same laboratory, irregular and unpredictable contamination is almost inevitable. It is thus best to devote an entire small room to the iodine work if dependable results are to be obtained.

In contrast to this trouble which is usually avoidable, once the patient has been administered any form of organic iodide, such as Lipiodol, Priodax, Skiodan, Diodrast or tetraiodophenolphthalein, binding of the iodine compound onto plasma proteins (11–13) renders the determination without value. Within a few hours, the PBI rises, often reaching a peak greater than 350 μgm. %. Depending upon the nature and amount of the particular substance plus the method of its administration, elevated PBI values have been observed for six months to three years in the absence of any genuine metabolic disorder (2, 14). No satisfactory method exists for separating these iodine-containing compounds from the true PBI, even the extraction of "thyroxine iodine" with butyl alcohol (15). It is highly recommended that information as to this type of contamination be widely disseminated so that samples for PBI analysis will be drawn before rather than after a diagnostic procedure using any organic iodide.

1) Precipitation and washing of plasma proteins. The blood sample may be collected with an anticoagulant or may be allowed to clot. The advantages of the former are speed in obtaining the plasma and a larger volume of fluid resulting, and of the latter that no foreign material need be added to the blood. No difference has been found between plasma and serum PBI and the routine in this laboratory has been to use oxalated bottles prepared in advance by evaporating 0.1 cc. of 20% potassium oxalate solution in each. The labels should be distinctive to avoid exchange with bottles from other laboratories. If serum is to be used, a paraffined tube is advantageous to allow easy clot retraction.

The procedure of washing the zinc proteinate precipitate by resuspension in distilled water and centrifugation has been found by both chemical and radioiodine tracer studies [4] to remove between 99.0 and 99.5% of the inorganic iodine present. At plasma inorganic I levels of 2 μgm. % (for normal humans) to 100 μgm. % (on some Lugol's-treated patients), the residual amounts (about 0.01 to 1.00 μgm. %) are not large enough to interfere. However, thyrotoxic patients being given five drops t.i.d. often are up to 300 μgm. %, and one woman receiving 20 drops t.i.d. rose to 1120 μgm. %. Although it is of questionable therapeutic value to use such extremely high iodine doses, the situation occasionally comes up and the laboratory must constantly be on the lookout. If all iodine therapy is stopped at least four days prior to drawing a sample for PBI, the plasma inorganic iodide will return nearly to the normal level, so that three washings are adequate. If I^{131} uptake by the thyroid gland is also to be measured, an even longer period of time will be required. It is felt that this type of precaution is better than increasing the number of washings of the protein precipitate.

2) Ashing.[5] It does not appear necessary to take any elaborate precautions to insure absolutely even distribution of the protein-sodium carbonate suspension on the wall of each tube. However, it is desirable to avoid leaving the entire contents at the bottom of the tube, since this may lead to

[3] Obtainable from the Finger Lakes Chemical Co., Etna, N. Y.

[4] The I^{131} used in this investigation was supplied by the Oak Ridge National Laboratory on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

[5] For the most consistent results possible, it has been found desirable to have a muffle furnace available for the exclusive use of the iodine laboratory. While awaiting replacement of a defective relay on our furnace, we attempted to share one in the institution's water laboratory. Random contaminations immediately became a serious problem until we could return to our own apparatus, although no source for the added iodide was ever found.

large bubble formation during the drying. Often such a bubble will rise through the entire tube, carrying ahead of it considerable protein material and causing a loss of PBI.

The ashing time and temperature have been found not critical, variations from two to three hours and from 575° to 625° C. causing insignificant differences in results. At temperatures above 650° C., softening of the Pyrex tubes occurs as well as loss of iodine. Although the ash in the recommended temperature range is gray rather than white, the incineration is adequate to put all of the organic iodine into extractable inorganic form. Below 500° C., there are so many products of incomplete ashing that the acid solution is yellow and substances are extracted which give a very high "iodine" value.

Each tube is used three or four times and then discarded in order to avoid excessive etching with possible loss of the sample.

3) Dissolving iodide from ash. The effervescence when the hydrochloric acid reacts with the ash is highly desirable in carrying off hydrogen sulfide formed from the sulfur-containing amino acids. If hydrogen sulfide remains when the ceric sulfate is added, insoluble ceric sulfide precipitates. Under the conditions described, this situation has never been observed.

4) Colorimetric determination of iodide present. All steps of this part of the procedure must be carried out with great care to reproduce accurately all conditions of volumes, time and temperature, since the measurement is made on a basis of iodide catalysis of a rate reaction. The actual conditions may be varied considerably, but those decided upon must be duplicated carefully from analysis to analysis if good agreement is to be had. This report has described the technique used at present in this laboratory on the experience of two years.

All mixing of solutions in this last phase must be accomplished by some technique which avoids both contamination and loss of solution, such as twirling each tube between the hands, flicking the bottom of each tube with several fingers in succession, or rapidly inverting each tube. The inversion or "flipping" procedure is preferred; this is carried out by grasping the colorimeter tube close to the open end between the thumb and first finger. The tube is inverted by raising it over one's head and then is rapidly returned. With a little practice one can achieve complete mixing of practically all miscible solutions in eight to 10 such inversions without losing a drop. This technique is especially valuable when the ceric sulfate solution must be mixed quickly and completely with the use of only one hand.

The method described earlier for carrying out the calculation was devised in order to rule out any possible effect of substances remaining in the final acid solution from the ash. Many nonvolatile compounds could conceivably be present in solution to alter the final colorimetric assay. Any such substance should affect the standard 0.04 μgm. of sodium iodide added to one aliquot as well as the unknown itself. Derivation of the computa-

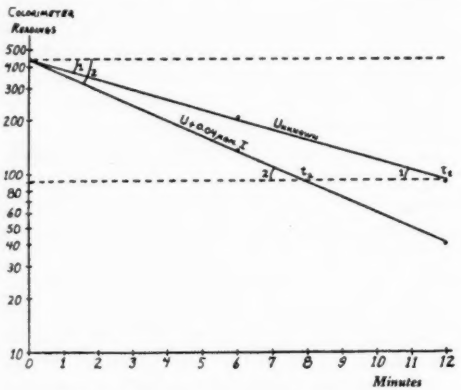

FIG. 1. METHOD OF PLOTTING RESULTS FOR CALCULATION OF PBI

$$\text{Tan} < 1 = \frac{\text{Initial colorimeter reading} - \text{final colorimeter reading}}{t_1}$$

This is an expression of the decolorization of the U sample. Since the assumption is being made that the decolorization is due entirely to I,

$$\frac{\text{Difference in colorimeter readings}}{t_1} = \text{U } \mu\text{gm. I in sample}$$

$$\text{Difference in colorimeter readings} = \text{U} \times t_1$$

In similar fashion:

$$\text{Tan} < 2 = \frac{\text{Difference in colorimeter readings}}{t_2} = \text{U} + 0.04\ \mu\text{gm. I}$$

$$\text{Difference in colorimeter readings} = (\text{U} + 0.04\ \mu\text{gm. I})t_2$$

Since the differences in colorimeter readings are identical,

$$\text{U} \times t_1 = (\text{U} + 0.04\ \mu\text{gm. I})t_2 = \text{U}t_2 + 0.04\ \mu\text{gm. I} \times t_2$$

$$\text{U}t_1 - \text{U}t_2 = 0.04\ \mu\text{gm. I} \times t_2$$

$$\text{U} = \frac{t_2 \times 0.04\ \mu\text{gm. I}}{t_1 - t_2}$$

The unknown also includes any iodide present in the reagents and a blank must be carried through as described in order to correct U for the blank.

TABLE I

Comparison of PBI methods on 57 clinical plasmas

Patient	Plasma PBI			Patient	Plasma PBI		
	Incin.	Distill.	Diff.		Incin.	Distill.	Diff.
	(1)	(2)	(1–2)		(1)	(2)	(1–2)
MR	1.0	1.1	−0.1	RS	6.4	6.8	−0.4
MN	1.8	1.6	+0.2	GM	6.8	7.4	−0.6
FMcE	2.2	2.2	0.0	OB	6.9	7.0	−0.1
DD	2.4	2.2	+0.2	EC	7.6	8.4	−0.8
JS	2.8	2.5	+0.3	WM	7.8	7.4	+0.4
LP	3.0	2.2	+0.8	SY	9.1	9.5	−0.4
WM	3.3	2.8	+0.5	EG	9.5	8.8	−0.7
JP	4.2	3.8	+0.4	JW	9.8	9.3	+0.5
KF	4.3	5.6	−1.3	VS	9.8	9.9	−0.1
MB	4.3	4.2	+0.1	AK	9.9	8.1	+1.8
ED	4.4	3.4	+1.0	EB	10.0	13.2	−3.2
HC	4.4	4.2	+0.2	SM	10.5	10.0	+0.5
TH	4.4	4.4	0.0	HB	11.1	12.3	−1.2
LB	4.4	4.6	−0.2	JW	11.4	10.1	+1.3
ID	4.4	4.8	−0.4	AB	12.0	9.0	+3.0
JM	4.6	3.7	+0.9	JZ	12.5	18.9	−6.4
OW	4.6	4.9	−0.3	JT	13.5	12.9	+0.6
CS	4.8	4.0	+0.8	JH	14.9	13.8	+1.1
NP	4.8	4.1	+0.7	JR	15.5	15.7	−0.2
GK	4.9	3.6	+1.3	JR	15.5	16.0	−0.5
EK	5.0	4.8	+0.2	JZ	16.0	17.8	−1.8
CM	5.1	4.6	+0.5	SY	16.2	16.7	−0.5
RM	5.3	6.2	−0.9	DR	19.5	16.4	+3.1
JS	5.5	4.5	+1.0	IB	19.9	17.9	+2.0
LP	5.5	6.0	−0.5	ST	21.3	20.4	+0.9
BT	5.6	4.4	−1.2	FF	21.5	28.0	−6.5
LN	5.7	4.9	−0.8	JE	27.8	26.3	+1.5
ES	5.9	4.7	+1.2	RQ	34.9	36.4	−1.5
MH	5.9	5.6	+0.3				

$$\chi^2 = \Sigma\left(\frac{(1-2)^2}{2}\right) = 11.096 \text{ for 56 degrees of freedom}$$

Therefore, there is much greater than 99% probability that the results are the same.

tion formula using the 0, 6 and 12 minute readings is contained in the detailed heading accompanying Figure 1. Table I summarizes a series of 57 comparisons which we have carried out on duplicate clinical plasma samples between this PBI procedure and the distillation technique described some time ago (16). A comparison of means would have little significance because of the wide range of PBI values (1 to 35 μgm. %). Chi square analysis of the results was therefore carried out and shows clearly that there is no significant difference between the two series.

That the unorthodox procedure of manipulation of the decolorization readings does not itself introduce a significant error when carefully done is demonstrated by 43 plottings of the identical colorimeter data carried out in the course of communicating the details of this procedure to interested laboratories. The mean of the calculations of the amount of iodine per colorimeter tube was 0.0386 μgm., with a standard deviation of 0.0004. Similarly good agreement has been obtained when different persons have plotted out the same colorimeter readings.

The customary method of calculating by setting up a curve of standards and interpreting colorimeter readings of unknown solutions from this curve is much simpler, but suffers from the possibility of the presence of some substance which would enhance the decolorization or interfere with it. The two methods of computation have been compared on several series of clinical samples; the standard curve technique was consistently higher, by 0.8 μgm. % using the 6-minute readings and 1.3 μgm. % using 12-minute readings. Recovery of the added 0.04 μgm. of standard iodide was 99.3% and 100.0%, respectively. No basis for this difference can be offered at present. It is obvious that use of the simpler form of calculation will necessitate upward revision of the values to be considered normal by about 1 μgm. %.

RECOVERY OF ADDED IODINE

As a test of the ability of the procedure to determine known amounts of iodine, standardized amounts of sodium iodide, thyroxine and thyroglobulin were added in triplicate to tubes containing precipitated and washed plasma proteins. Drying, ashing, HCl and H_2SO_4 elution and colorimetric determination of iodide present then followed. The results, shown in Table II, demonstrate adequate recovery of these substances in physiologically important amounts.

TABLE II

Recovery of iodine-containing substances added to precipitated and washed proteins

Substance	Amount I added	Amount I recovered
	μgm. %	%
NaI	2.0	97.5
	4.5	99.0
	5.0	108.9
	10.0	101.6
	20.0	102.3
Thyroxine	3.6	90.6
	7.7	98.0
	10.0	96.8
Thyroglobulin	2.5	90.9
	5.0	107.3
	15.0	94.2

RESULTS WITH THE DETERMINATION

This procedure has been available to the staff of the University Hospitals for nearly two years. On the basis of the first year's experience, the technique was extensively revised during the summer of 1949. In the year since that time, 942 determinations have been carried out on 694 patients; the distribution of these is shown in Figure 2. A

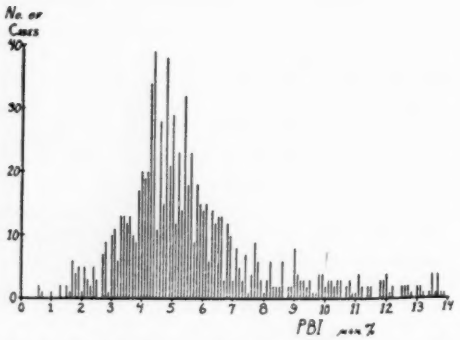

FIG. 2. DISTRIBUTION OF ONE YEAR'S DETERMINATIONS OF PBI ON CLINICAL PLASMA SAMPLES

In all, 942 results were obtained, but 50 values above 14 μgm. % have been omitted from the chart, since these were scattered up to 45 μgm. %.

clustering of values between 3 and 7 μgm. % is apparent, but no sharp separations are present between "normal" and either hypo- or hyperthyroid individuals. From a physiological standpoint, such overlapping would be expected on the basis of all studies of normalcy. From the clinical point of view, it is important to establish some limits beyond which thyroid dysfunction can reasonably by expected.

In order to accomplish this, a group of samples has been assembled in Table III from medical students, resident staff, technicians and faculty, each person overtly appearing to be euthyroid. The table shows that a range of PBI values from 3.4 to 8.0 μgm. %, with a mean of 5.1, was obtained on 40 medical students who had BMR's between − 15 and + 15. A smaller group of 27 apparently normal individuals (staff, visitors, etc.) gave an identical mean, although no basal metabolism values were available for standardization. In view of these findings, it is probable that the range of normal, usually considered to be 4.0 to 8.0 μgm. %, must be extended downwards to 3.5 μgm. %. This conclusion is, of course, based partially on acceptance of the validity of the BMR. Since the usual error in this determination is on the plus side, one might argue that this error had operated in the three subjects whose PBI values were below 3.9 μgm. % and that they actually should be considered myxedematous. No answer to this is possible, except that no genuine symptoms of hypothyroidism were apparent in any of these individuals. It will require a great many carefully controlled determinations in the borderline PBI zone of 3.5–4.0 to settle this physiological as well as chemical problem.

TABLE III

PBI values on 68 euthyroid subjects

Subjects	BMR	PBI		
	Range	Mean	Range	σ
		μgm. %	μgm. %	
40 Medical students	+15 to −15	5.1	3.4–8.0	1.12
28 Miscellaneous	unknown	5.1	3.6–6.6	0.79
Entire series of 68		5.1	3.4–8.0	1.00

It is highly possible that the upper limit should be 7.5, bringing the entire euthyroid group close to the range of 3.5–7.0 μgm. % as suggested by Riggs (2). The problems of overlap, as far as the PBI is involved, seem to be intensified in the lower, myxedema, portion, with very few euthyroid individuals above 7.5 or thyrotoxic below 8.5 μgm. %.

The histories on 86 of the patients on whom plasma PBI's had been run also contained results of BMR and I^{131} uptake determinations. The data for PBI and BMR have been plotted in Figure 3, together with the overall clinical evaluation of each patient, proven by surgery, pathology or therapeutic response. The area between a BMR of + 15 and − 15 and between 3.5 and 8.0 for PBI has been marked off as normal. It can be seen that one value in the myxedema group would be considered normal (at 3.5 μgm. %), one thyrotoxic was well in the normal range, and one very high value (14.3 μgm. %) was obtained on a subject with a non-toxic, diffusely enlarged goiter. In contrast, several BMR determinations in each category were at considerable variance with the general clinical opinion. The greater reliability

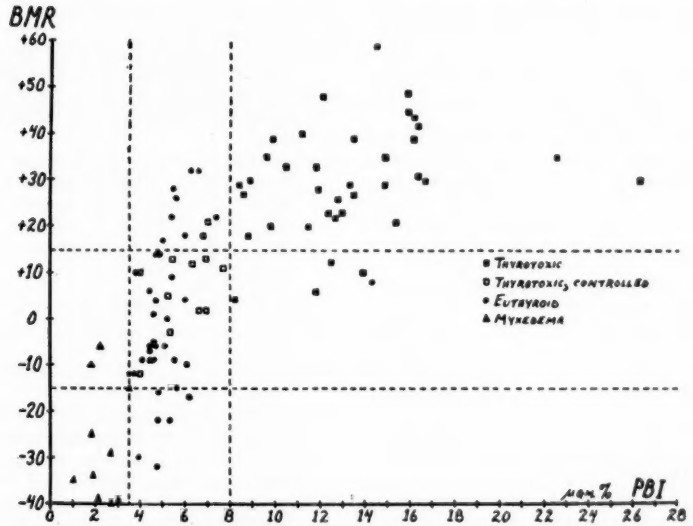

FIG. 3. COMPARISON OF PBI, BMR AND ENTIRE CLINICAL EVALUATION OF 86 PATIENTS

The 12 values represented by hollow squares were performed on previously thyrotoxic subjects, following successful management with surgery, I^{131}, or propylthiouracil. All patients had been on therapy at least one month, which should be an adequate length of time for the metabolic rate to return to normal (17).

of the PBI over the BMR has previously been noted by several investigators (2, 3, 18) and may be attributed, at least in part, to the greater objectivity of the chemical determination.

In discussing the advantages of this procedure, it may simply be stated that, in general, distillation techniques, although having a wider application because of greater flexibility, require such close attention during the digestion and distillation steps as to be poorly suited for routine clinical evaluation. On the other hand, incineration can be automatically regulated, making the final colorimetry the only stage requiring personal concentration. No attempt has been made in this laboratory to determine the maximum number of determinations possible per day, since successful routine analysis depends much more upon consistent performance week after week. It may conservatively be estimated that 15–20 samples can be analyzed in duplicate every two days by a single individual.

SUMMARY

1. A method has been presented for determination of plasma or serum protein-bound iodine (PBI) using high-temperature incineration in the presence of alkali. The distillation step, requiring close individual attention, has been eliminated. The final determination of inorganic iodide is performed colorimetrically by measuring the catalysis of arsenious acid decolorization of a ceric sulfate solution.

2. Data are given for considering the range of normal to be 3.5 to 8 μgm. %.

3. Examination of two years' experience with this PBI analysis shows it to be an objective test of greater dependability than the BMR.

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STUDIES OF THE ANTIDIURESIS OF QUIET STANDING: THE IMPORTANCE OF CHANGES IN PLASMA VOLUME AND GLOMERULAR FILTRATION RATE [1]

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INTRODUCTION

It has long been known that when a man stands motionless, urine flow (1–13) and the excretion of chloride (2, 3, 5, 7, 11) and of sodium (5, 13) decrease markedly. Simultaneously, the plasma contracts in volume and increases in concentration (8, 10, 14–17), while the blood flow through the kidneys (9, 10, 18, 19) and other abdominal viscera (20) diminishes, and the rate of glomerular filtration of plasma and its contained sodium is reduced (6, 9, 10, 13, 18, 21).

There is some evidence that release of the posterior pituitary antidiuretic hormone may be responsible for the retention of water (11) but the mechanism of the retention of sodium and the stimulus or stimuli initiating it remain obscure. Some renal physiologists would place greatest emphasis on the reduction in glomerular filtration rate which occurs during standing, and the resulting decreased load of sodium presented to the tubules (5, 22, 23). It is not likely that an increase in the renal venous pressure (24, 25) plays an important role in the antidiuresis of quiet standing, because when a subject is tilted from the supine to the erect position, pressure in the inferior vena cava at the level of the kidneys does not rise to the level reported by Blake and associates (25) to cause a reduction in the urine flow, and the renal arterio-venous pressure gradient does not change materially (26).

It has also been suggested that the reduction of total blood volume initiates a chain of events which culminates in decreased excretion of sodium (27–29). This might be accomplished through changes in glomerular filtration and/or tubular reabsorption of sodium. Some workers believe that this mechanism may operate in such diverse situations as hemorrhage, hypoproteinemic edema and congestive heart failure, as well as in quiet standing (28, 30).

The present studies were undertaken to investigate the relationship of changes in total plasma volume and glomerular filtration rate to the renal excretion of sodium during quiet standing.

METHODS

All subjects were healthy young men, presumably able to empty their bladders without catheterization. Relatively high urine flows and long periods minimized any errors in collection. A fairly constant rate of excretion of sodium was achieved by the ingestion of 200 ml. of hypotonic (0.2%) saline solution per hour for several hours (31), and by preliminary rest supine in bed for at least one hour, before observations were begun. Subjects continued to drink saline at the same rate during the experimental period. In five experiments (Nos. 1, 10, 11, 13, 14) the glomerular filtration was estimated from the clearance of mannitol, using the single injection method with a falling plasma concentration (32). Previous observations had demonstrated that one could maintain a constant diuresis of salt and water for several hours under these conditions (33). In six cases (Nos. 4–6, 8, 9, 15) a constant infusion of mannitol was used, and in the remaining experiments a constant infusion of inulin. Renal plasma flow was calculated from the clearance of sodium para-aminohippurate administered as a constant infusion in normal saline. Mannitol in serum and urine was analyzed by the method of Smith (34), modified according to Elkinton (35). Inulin and para-aminohippurate were determined using methods described by Goldring and Chasis (36). Concentrations of sodium in serum and urine were measured with a flame photometer. Acute changes in plasma volume (PV) were calculated

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TABLE I

Changes in sodium excretion, plasma volume and renal function with quiet standing

Experiment no. and subject	Period	Duration	Urine flow	Sodium excretion	Mannitol clearance	Inulin clearance	Average serum Na	Sodium filtered	$\frac{E^*}{F}$ Na	% Change in plasma volume from control	PAH clearance	Mannitol excreted
		min.	*ml./min.*	*mEq./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*mEq./L*	*mEq./min. per 1.73 m²*			*ml./min. per 1.73 m²*	*mM/min.*
1 ERP	1-supine	67	4.18	0.159	85.6	—	136.5	11.5	0.014	0	—	0.43
	2-standing	60	1.97	0.0714	76.0	—	136.5	10.21	0.007	−13	—	0.25
	3-supine	61	0.74	0.0197	61.3	—	136.5	8.27	0.002	− 4	—	0.14
2 FHE	1-supine	30	8.1	0.266	—	108.3	144.0	15.5	0.017	0	599	—
	2-supine	32	5.0	0.305	—	99.4	144.5	14.3	0.021	− 6	556	—
	3-standing	25	1.6	0.218	—	85.0	145.0	12.2	0.018	−10	526	—
	4-supine	38	2.4	0.252	—	86.0	144.5	12.3	0.020	− 8	589	—
3 ASR	1-supine	30	3.90	0.181	—	—	—	—	—	—	722	—
	2-supine	30	3.93	0.155	—	—	—	—	—	0	585	—
	3-standing	30	1.55	0.096	—	—	—	—	—	—	526	—
	4-standing	30	0.57	0.073	—	—	—	—	—	− 7	548	—
	5-supine	30	0.77	0.081	—	—	—	—	—	—	743	—
	6-supine	31	1.16	0.090	—	—	—	—	—	0	787	—

* $\frac{\text{Sodium excreted}}{\text{Sodium filtered}}$.

from changes in the hemoglobin (Hgb) and hematocrit (Hct) of venous blood drawn without stasis, using formula:

$$\frac{PV_2}{PV_1} = \frac{Hgb_1}{Hgb_2} \times \frac{1 - Hct_2}{1 - Hct_1},$$

where the numbers in subscript indicate the values at the beginning and the end of a given period, respectively. Samples of blood for analysis were drawn at the beginning and at the end of each clearance period. The rate of filtration of sodium was calculated as the product of the average concentration of serum sodium and glomerular filtration rate, corrected for an assumed serum water concentration of .93 and a Donnan factor for sodium of 0.95. The fraction of filtered sodium rejected by the tubules and appearing in the urine was calculated as the fraction:

$$\frac{\text{Na excreted}}{\text{Na filtered}}, \text{ (E/F Na)}.$$

RESULTS

Quiet Standing Alone (Table I):

In three subjects the renal effects of standing quietly for 25 to 60 minutes were quite typical of

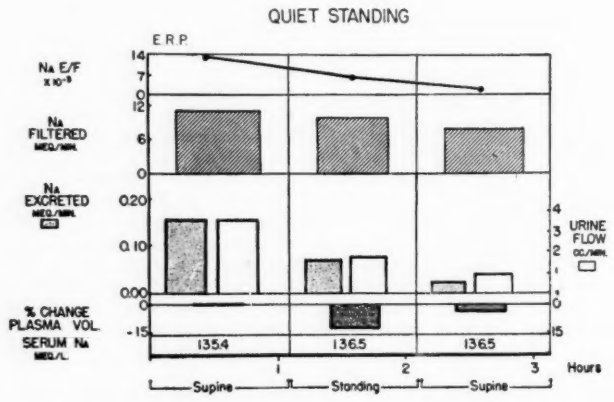

FIG. 1. EFFECT OF ONE HOUR OF QUIET STANDING

Note prolonged antidiuresis and the reduction in sodium excretion, associated with a significant contraction of plasma volume.

TABLE II

Effect of infusions of serum albumin during quiet standing

Experiment no. and subject	Period	Duration	Urine flow	Sodium excretion	Mannitol clearance	Inulin clearance	Average serum Na	Sodium filtered	$\frac{E^*}{F}$ Na	% Change in plasma volume from control	PAH clearance	Mannitol excreted
		min.	*ml./min.*	*mEq./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*mEq./L*	*mEq./min. per 1.73 m²*			*ml./min. per 1.73 m²*	*mM/min.*
4 GG	1-supine	57	9.39	0.150	86.5	—	132.5	11.4	0.013	0	524	0.52
	2-standing	49	4.59	0.103	79.6	—	134.7	10.6	0.010	+ 6	474	0.43
	300 ml. 25% albumin in 34 min.											
	3-supine	41	4.15	0.043	73.4	—	135.9	9.9	0.004	+26	528	0.37
5 IT	1-supine	33	5.15	0.223	79.0	—	141.2	11.0	0.020	0	498	0.53
	2-standing	44	4.54	0.194	72.0	—	139.6	10.0	0.019	+ 4	479	0.52
	750 ml. 4% albumin in 34 min.											
	3-standing	38	2.71	0.012	68.3	—	138.9	9.4	0.012	+ 3	462	0.54
6 ASR	1-supine	35	3.51	0.180	92.0	—	—	12.9†	0.014	0	—	0.52
	2-supine	33	5.24	0.283	97.0	—	—	13.6	0.021	0	—	0.56
	3-standing	37	3.51	0.208	82.3	—	—	11.5	0.018	+ 3	—	0.47
	750 ml. 4% albumin in 30 min.											
	4-standing	32	2.75	0.182	84.5	—	—	11.8	0.015	+ 7	—	0.48
	5-supine	37	1.76	0.099	74.5	—	—	10.4	0.010	+10	—	0.42
7 FHE	1-supine	25	7.40	0.451	—	96	145.8	13.9	0.032	0	625	—
	2-supine	32	4.37	0.533	—	92	145.0	13.2	0.040	+ 4	642	—
	3-supine	30	5.33	0.500	—	90	144.5	12.9	0.039	+ 6	675	—
	4-standing	42	2.02	0.240	—	83	145.0	11.9	0.020	+ 7	578	—
	750 ml. 4% albumin in 35 min.											
	5-standing	32	1.41	0.109	—	99.5	144.8	14.3	0.008	+15	730	—

* $\frac{\text{Sodium excreted}}{\text{Sodium filtered}}$.

† Serum concentration of Na assumed to be 140 mEq./L.

those described by others (Figure 1). Renal plasma flow, the filtration and excretion of water and sodium, and the tubular rejection of sodium (E/F Na) all decreased. These changes lasted for some time after resumption of the supine position. Plasma volume decreased by 4 to 13%, but [as previously reported for serum chloride and bicarbonate (14)] the concentration of serum sodium did not change significantly.

Quiet Standing with Albumin Infusion (Table II):

To determine whether this sequence of events would be altered if the contraction of plasma volume were prevented, four subjects were given infusions of salt-poor human serum albumin [6] when they stood up. In three experiments, 750 ml. of isosmotic albumin (4% solution in normal saline) were infused, while in one case, 300 ml. of a 25% solution were injected. The infusions were given over a period of 30 to 35 minutes. A typical result is shown in Figure 2. Although in all cases total plasma volume increased slightly, in no instance was the antidiuresis of salt and water, which ordinarily accompanies quiet standing, prevented; and in every case a fall in the glomerular filtration of sodium and in E/F Na occurred. In experiment 7 (Figure 2), the excretion of sodium continued to decline during the last period despite an increase above control levels in both plasma volume and filtered sodium. Changes in renal plasma flow tended to parallel changes in the rate of glomerular filtration.

[6] Serum albumin used in this study was prepared by the American Red Cross from blood of volunteer donors. The conclusions are those of the authors and do not necessarily reflect the policy of the National Blood Program of the American Red Cross.

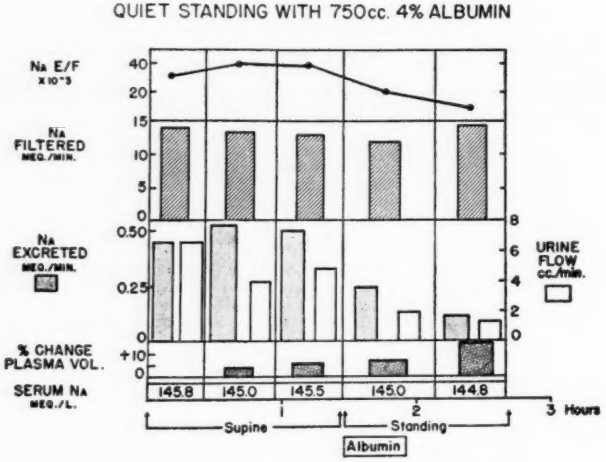

FIG. 2

Albumin infusion prevented the expected contraction of plasma volume during the period of quiet standing, but did not prevent the usual antidiuresis. Note that excretion of sodium and water continued to fall in the last period, although the rate of sodium filtration increased.

Effects of Infusions of Hypertonic Saline (Tables III and IV):

It is well known that the sodium diuresis following infusions of hypertonic saline is accompanied by an increase in the concentration of sodium in the serum and expansion of the volume of the plasma and extracellular fluid. In two experiments (Table III), infusions of hypertonic saline (6%) given to recumbent subjects sharply augmented the excretion of sodium, with (No. 9) or

TABLE III

Effect of rapid infusions of hypertonic sodium chloride in supine subjects

Experiment no. and subject	Period	Duration	Urine flow	Sodium excretion	Mannitol clearance	Inulin clearance	Average serum Na	Sodium filtered	$\frac{E^*}{F}$ Na	% Change in plasma volume from control	PAH clearance	Mannitol excreted
		min.	*ml./min.*	*mEq./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*mEq./L*	*mEq./min. per 1.73 m²*			*ml./min. per 1.73 m²*	*mM/min.*
8 McG	1-supine	39	7.3	0.224	119	—	132.0	15.55	0.014	—	485	0.62
	2-supine	62	9.2	0.282	129	—	135.9	17.35	0.016	—	536	0.68
	3-supine 400 ml. 6% saline in 23 min.	47	6.6	0.870	120	—	141.6	16.94	0.051	—	444	0.62
	4-supine	48	7.4	1.11	118	—	143.8	16.80	0.066	—	—	0.62
9 BR	1-supine	40	7.13	0.135	120	—	131.7	15.65	0.009	—	636	0.48
	2-supine	39	10.40	0.125	107	—	133.5	14.14	0.009	—	651	0.43
	3-supine 400 ml. 6% saline in 39 min.	52	3.75	0.287	116	—	139.0	15.95	0.018	—	725	0.47
	4-supine	34	3.92	0.520	128	—	141.7	17.95	0.029	—	902	0.52

* $\frac{\text{Sodium excreted}}{\text{Sodium filtered}}$.

TABLE IV

Effect of infusions of hypertonic sodium chloride during quiet standing

Experiment no. and subject	Period	Duration	Urine flow	Sodium excretion	Mannitol clearance	Inulin clearance	Average serum Na	Sodium filtered	$\frac{E^*}{F}$ Na	% Change in plasma volume from control	PAH clearance	Mannitol excreted
		min.	*ml./min.*	*mEq./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*ml./min. per 1.73 m²*	*mEq./L*	*mEq./min. per 1.73 m²*			*ml./min. per 1.73 m²*	*mM/min.*
10 ASR	1-supine	49	3.27	0.296	108.0	—	140.5	14.95	0.020	0	—	0.31
	2-standing	55	1.36	0.053	70.6	—	136.5	9.50	0.006	−21	—	0.18
	3-standing 400 ml. 3% saline in 30 min.	63	0.75	0.023	64.9	—	142.0	9.10	0.003	+ 1	—	0.14
11 ERP	1-supine	50	4.00	0.438	88.3	—	137.2	12.1	0.036	—	—	0.50
	2-standing 400 ml. 4% saline in 25 min.	75	2.37	0.266	67.0	—	142.0	9.5	0.028	—	—	0.23
	3-supine	51	2.12	0.315	77.0	—	141.9	10.9	0.029	—	—	0.13
12 FHE	1-supine	31	4.68	0.499	—	97.0	146.0	14.05	0.035	0	646	—
	2-supine	39	5.57	0.554	—	94.0	145.6	13.58	0.040	− 1	638	—
	3-standing 400 ml. 6% saline in 35 min.	45	2.04	0.443	—	88.0	149.7	13.05	0.034	0	652	—
	4-standing	30.5	1.72	0.439	—	83.0	152.1	12.53	0.035	0	584	—
13 AVG	1-supine	48	5.10	0.666	98.0	—	136.5	13.3	0.050	—	—	0.28
	2-standing 400 ml. 6% saline in 25 min.	71	3.66	0.622	74.5	—	143.0	10.6	0.059	—	—	0.21
	3-standing	55	2.18	0.412	49.0	—	143.5	7.0	0.059	—	—	0.20
14 FHE	1-supine	54	5.65	0.627	94.5	—	139.1	13.1	0.046	—	—	0.54
	2-standing 400 ml. 6% saline in 37 min.	63	4.92	0.743	86.3	—	146.5	12.6	0.059	—	—	0.32
	3-supine	53	4.15	1.00	97.8	—	152.5	14.9	0.067	—	—	0.24
15 DM	1-supine	40	2.50	0.210	100.0	—	138.9	13.80	0.015	0		0.62
	2-supine	44	3.07	0.238	99.0	—	137.6	13.52	0.017	0	432	0.55
	3-standing 400 ml. 6% saline in 26 min.	41	4.83	0.468	92.0	—	140.0	12.80	0.036	+16	522 613	0.49
	4-standing	39	2.82	0.356	100.0	—	145.1	14.40	0.025	+11	437	0.50
16 ASR	1-supine	30	3.80	0.200	—	100.	146.0	16.60	0.014	0	774	—
	2-supine	31	3.64	0.259	—	89.	145.5	14.70	0.020	− 2.5	697	—
	3-standing 400 ml. 7½% saline in 40 min.	45	3.04	0.532	—	105.	153.5	18.30	0.033	+18	865	—
	4-standing	29	3.:0	0.852	—	108.	157.0	19.1	0.051	+17	940	—

* $\frac{\text{Sodium excreted}}{\text{Sodium filtered}}$.

without (No. 8) increasing appreciably the glomerular filtration of sodium or the renal plasma flow. In seven other experiments (Table IV), therefore, the hypertonic saline was infused during a period of standing, in an attempt to observe how the plasma volume, the concentration of sodium in the serum, and the rate of glomerular filtration of sodium were related to the excretion of sodium when large loads of salt were administered to subjects assuming the erect posture.

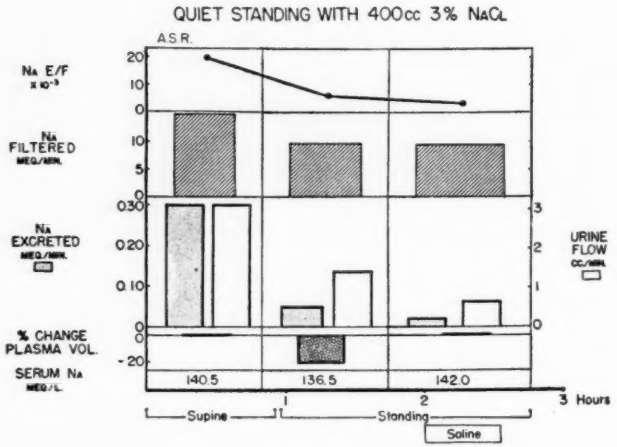

FIG. 3

The infusion restored the plasma volume to normal and prevented further significant change in sodium filtration. Despite this, urine flow and sodium excretion continued to diminish.

The rise in concentration of serum sodium was significant in all cases, but in only one was it adequate actually to increase the rate at which sodium was filtered during the period of standing. Plasma volume, calculated in some of these experiments, usually increased. The excretion of sodium declined in four instances and increased in three. As might be expected, the excretion of sodium seemed roughly proportional to the size of the salt load.

Subject ASR (No. 10, Table IV, and Figure 3) received 12 grams of salt as 400 ml. of 3% saline

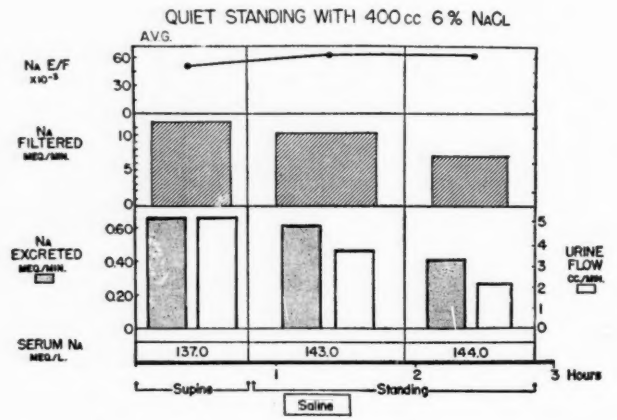

FIG. 4

The infusion did not prevent the reduction in urine flow and sodium filtration and excretion during the periods of quiet standing, but it did provoke the tubules to reject a larger fraction of the filtered sodium.

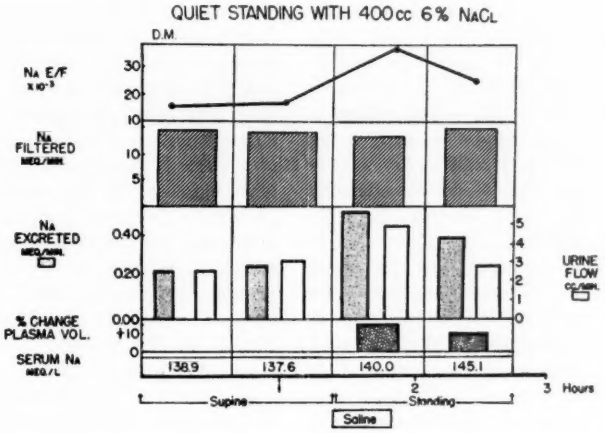

FIG. 5

Rapid administration of 24 grams of salt markedly increased plasma volume and sodium and water excretion during quiet standing, in the absence of significant changes in sodium filtration.

after a period of standing. This slightly increased the serum sodium and re-expanded the plasma volume to its control value. There was a further decline in the glomerular filtration of sodium of questionable significance. Despite this, sodium excretion decreased markedly, because the tubules reabsorbed a greater fraction of the filtered load of sodium presented to them. In an experiment in which 400 ml. of 4% saline were given (No. 11, Table IV), essentially the same results were obtained.

In two experiments administration of 400 ml. of 6% saline did not prevent a decline in the glomerular filtration or in the excretion of sodium, but in AVG (No. 13, Table IV; Figure 4) it did cause the tubules to reject a slightly greater percentage of the filtered sodium.

In two other subjects (Nos. 14 and 15, Table IV; Figure 5), one of whom (No. 14) had ingested 24 grams of salt during the previous 24 hours, the same amount of 6% saline proved adequate completely to reverse the antidiuresis of quiet standing. Excretion of sodium actually rose considerably, together with an expansion of the plasma volume, and without any increase in the calculated rate of glomerular filtration of sodium.

Finally, in one case (No. 16), 400 ml. of 7½% saline infused over 40 minutes not only increased the excretion of sodium during a period of quiet standing, but increased the glomerular filtration rate and renal plasma flow, as well as the plasma volume and the concentration of serum sodium.

DISCUSSION

These data confirm the earlier findings of others that a contraction of plasma volume, a decrease in renal clearances, and a diminished excretion of salt and water occur in the erect position. The fall in salt excretion during upright-tilting experiments, reported by Brun, Knudsen and Raaschou (9), was attributed by them to the fact that their subjects were in a state of pronounced negative chloride balance. This was not the case in the present studies, in which dilute saline was ingested for several hours preceding the test (31).

Infusions of 4% albumin do not influence the rate at which sodium is excreted by supine subjects (37, 38), although there is conflicting evidence concerning the effect of more concentrated solutions (33, 37, 39–41). It would appear, therefore, from the experiments of Table II that the antidiuresis of quiet standing is not necessarily dependent upon a contraction of the total plasma volume, since excretion of salt and water diminished during standing, even when plasma volume was slightly expanded by rapid infusions of al-

bumin. However, it must be emphasized that such infusions control only the *total volume* of the circulating blood and do not necessarily alter its *distribution* during quiet standing. Blood pooled in certain peripheral areas may not contribute to the effectiveness of the circulation, and in this sense the "effective blood volume" (42) may be reduced even when the total blood volume remains unchanged. Standing erect decreases both the total and effective volumes of the circulation by increasing the capillary filtration and the pooling of blood in the limbs. When these disturbances are minimized by immersing the body in water up to the level of the heart or by applying pneumatic occluding cuffs to the legs, the antidiuresis of the erect posture is modified (8, 12). It would seem, therefore, that if the kidneys react to changes in some function of the volume of the circulating blood when a man stands erect, they must react more to changes in its effective distribution than in its total volume.

The view that the antidiuresis of quiet standing is mediated primarily through a change in renal hemodynamics was first advanced by Hooker, who invoked the narrowed pulse pressure in the renal artery as a cause for diminished urine flow (4, 43). White, Rosen, Fischer, and Wood (5) ascribed the increase in the excretion of water and electrolytes during recumbency mainly to an increase in glomerular filtration rate. On the other hand, Ni and Rehberg (6), using the clearance of exogenous creatinine as a measure of filtration rate, came to the conclusion that postural changes in urine flow resulted from changes in both tubular reabsorption and glomerular filtration. In the present experiments, reductions in the urinary excretion of sodium during quiet standing were usually accompanied by significant alterations in both the filtration rate and the tubular rejection fraction of sodium (E/F Na). However, when infusions of albumin and hypertonic saline were given to subjects standing erect, a fall in sodium excretion was not always accompanied by a fall in the glomerular filtration of sodium (No. 7), nor did a small decrease in the filtration of sodium always result in diminished excretion of sodium (Nos. 14, 15).

These experiments appear to shed light on the nature of the renal response to loads of hypertonic saline, as well as to the upright posture. It has been suggested that the urinary excretion of sodium is normally regulated through fluctuations in its glomerular filtration (22, 23, 44). Selkurt and Post (45) reported that when dogs are infused with hypertonic saline, the excretion of sodium varies as the filtered load, and Dean and McCance (46) concluded that during hypertonic saline infusions in human subjects, "changes in glomerular filtration must underlie, and in part determine, the observed changes in urine flow." It is therefore noteworthy that in experiments 8, 14, and 15, when 6% saline was infused, the excretion of sodium increased without a concomitant increase in filtered load (but not without an increase in plasma volume).

The use of mannitol in these experiments may be questioned on the grounds that since excreted mannitol may sweep salt into the urine (22, 44, 47, 48) sodium excretion might necessarily appear to be related to the excretion, and hence the filtration of mannitol. This consideration enhances the significance of such discordant changes in the filtration and excretion of sodium as were observed in experiments 8, 14, and 15. Nevertheless, even under the strenuous conditions of these experiments it was difficult to change greatly the excretion of sodium in a direction opposite to simultaneous changes in sodium filtration. This does not necessarily prove that excretion is dependent upon filtration, since they may both be related through a third controlling factor.

Except in cases where the changes are very large, the errors inherent in present measurements of glomerular filtration prevent any accurate apportionment of responsibility to tubules or glomeruli for alterations in sodium excretion. Each milliliter of glomerular filtrate contains approximately 0.14 mEq. of sodium. An unavoidable 5% error in the estimation of glomerular filtration may therefore involve as much as 1 mEq./min. of sodium.

The renal response to salt loads is conditioned in part by the rise in concentration of sodium in the extracellular fluid. When infusions of hypertonic saline are given to thirsted subjects, the rate of sodium excretion rises, but drops sharply when the serum sodium is suddenly restored to normal by the ingestion of a large quantity of water (49). Some workers have concluded that when an infusion of hypertonic saline is given,

"changes in sodium excretion are invoked by differences between extracellular and intracellular osmotic pressures" (50). The present studies show that even when the concentration of sodium in the serum rises significantly, it is sometimes possible to prevent or reverse the usual renal response by standing upright. A similar effect from abdominal compression has been reported recently (51).

The present data are consistent with the pluralistic view that alterations in both the composition and the effective distribution of the circulating plasma are important in initiating acute renal adjustments in sodium excretion. These adjustments may be accomplished by appropriate changes in the glomerular filtration or the tubular reabsorption of sodium, or both.

CONCLUSIONS

1. The antidiuretic reaction to standing does not require a contraction of the total plasma volume.

2. It is possible to reverse this reaction by administration of hypertonic saline without increasing the rate at which sodium is filtered; but in so doing, the plasma volume is expanded.

3. The renal response to sodium loads under these conditions is not dependent solely upon alterations in the glomerular filtration of sodium or in the concentration of sodium in the serum.

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STUDIES IN GLYCINE-2-C^{14} METABOLISM IN MAN. I. THE PULMONARY EXCRETION OF $C^{14}O_2$[1]

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INTRODUCTION

There has been considerable discussion regarding the use of C^{14} in clinical investigation, particularly from the standpoint of retention in the body of a long lived (5600 years) radioactive isotope. There is evidence from animal investigations that following the intravenous injection of simple C^{14} labeled compounds occurring in normal biochemical processes most of the radioactivity will be excreted as $C^{14}O_2$ within a few days (1–5). The rate of excretion in mice via the lungs and the rate of loss of C^{14} from the soft tissues and bone when this isotope is administered as glycine-2-C^{14} has been determined; most of the radioactivity was eliminated in the breath in the first two days and the slowest tissue component was found to have a half time of approximately 10 days (6). There is no method available for extrapolation of these animal data to man. The present study was designed to measure the rate of pulmonary excretion of C^{14} as $C^{14}O_2$ when administered as glycine-2-C^{14}.

METHODS

Methyl labeled glycine was prepared by chlorination and amination of acetic acid-2-C^{14} and had a specific activity of 12.2 microcuries/mg. of glycine (7). A series of four patients was given approximately 100 microcuries of glycine-2-C^{14} (8 mg.) intravenously in 10 ml. distilled water for the purpose of the determination of the red blood cell life (8), since previous investigations with radio-iron had indicated that many patients with polycythemia and chronic leukemia might have red blood cells with a shorter than normal life span (9). Frequent breath samples were taken by a device (see Figure 1) designed to collect the expired air in a rubber balloon for a measured period of time. The balloon was transferred to a bubbler system (see Figure 2) and the expired air drawn through a 1N sodium hydroxide solution. The carbon dioxide thus absorbed was precipitated as $BaCO_3$ by NH_4NO_3 and $BaCl_2$. The barium carbonate was filtered, dried at 110° C and weighed to determine the amount of CO_2 excreted per minute. The samples were counted with a proportional counter[2] where the specific activity was greater than 10 dis./min./mg. $BaCO_3$; those with a lower activity were measured in an ionization chamber. These instruments were calibrated with the current Oak Ridge C^{14} standard millicurie by specially prepared samples.

Inasmuch as the rate of pulmonary excretion of C^{14} when administered in this form was not known at the start of these studies, the patients were placed in an oxygen tent although no oxygen was supplied, so that the expired air would pass through the soda-lime filter and thus absorb the carbon dioxide. In addition a vacuum pump continually exhausted the air from inside the tent to the outside of the building. These precautions were maintained for approximately 36 hours.

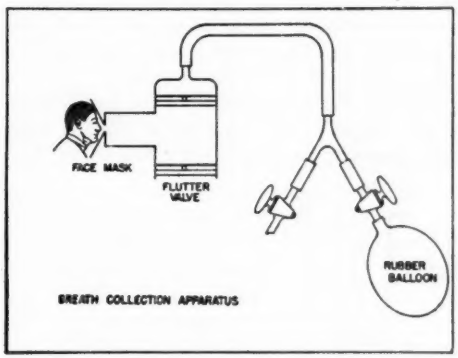

FIG. 1.
BREATH COLLECTION APPARATUS
(Note: not drawn to scale.)

RESULTS

Figure 3 shows the specific activity as a function of time of the expired carbon dioxide as $BaCO_3$. The maximum specific activity of the expired breath occurred within the first hour following the injection. A measurable amount of radio-

[1] This work was supported in part by the Atomic Energy Commission.

[2] Nucleometer—Manufactured by Radiation Counter Laboratory, Chicago, Illinois.

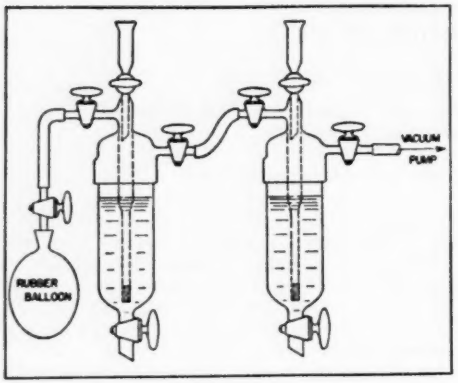

FIG. 2.
APPARATUS FOR CARBON DIOXIDE ABSORPTION

activity was present within the first 10 minutes following the injection. Figure 4 shows the calculated cumulative C^{14} excretion as determined from the amount of CO_2 excreted per minute and the rate constants obtained from Figure 1.

The rate of excretion of C^{14} when administered in this manner may be described in terms of three rate processes: the first having a half time of two to four hours; the second, 16 to 40 hours; and the third, seven to 14 days. Table I shows the half times and the percentages of C^{14} excreted through each of the three rate processes for the four patients.

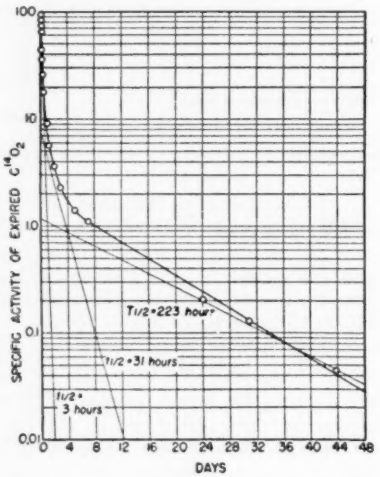

FIG. 3.
THE SPECIFIC ACTIVITY OF THE EXPIRED BREATH AS A FUNCTION OF TIME, IN PATIENT 1, IN DISINTEGRATIONS/MIN./MG. OF $BaCO_3$

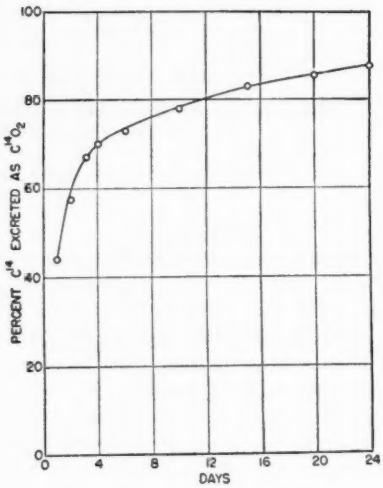

FIG. 4.
THE CUMULATIVE C^{14} EXCRETION AS $C^{14}O_2$ IN PATIENT 1

The cumulative excretion after the 24th day is not shown, but by the 45th day it will amount to approximately 92 per cent of the injected dose.

DISCUSSION

The cumulative excretion of C^{14} is so rapid that in Patient 1 at the end of one day approximately 40 per cent has been eliminated by this route, and by the end of five days 60 per cent; by 20 days, approximately 83 per cent (see Figure 4). Furthermore, in all four patients by the end of 40 to 75 days there was no significant measurable activity in the breath; *i.e.*, less than 0.04 dis./

TABLE I

The per cent of the administered dose excreted and half time for each of the three rate processes

Patient	T_1 (1/2)	Per cent	T_2 (1/2)	Per cent	T_3 (1/2)	Per cent	Total per cent excreted
	hours		*hours*		*hours*		
1	3	36	31.2	27	223	29	92
2	2.7	32	40	13	163	38	83
3	2.5	13	36	21	336	52	86
4	3.3	22	16	17	240	49	88

min./mg. $BaCO_3$. It is probable that there is a slower component, the radioactivity of which could be detected by more sensitive methods (10, 11); however, this could represent only a small fraction of the administered dose.

The relation of these three different rate processes to known biochemical processes is not known. However, the measurement of the uptake of C^{14} in the plasma proteins and their turnover time suggests that the slowest component may represent the rate constant for protein synthesis and turnover (12).

The finding that the slowest measured rate of excretion is of the same order of magnitude in man as in mice should permit the extrapolation to man of animal investigations with similar C^{14} labeled compounds. This may allow the use of such animal data as the basis for the determination of the hazard of using C^{14} in clinical investigation. In addition to the 83 to 92 per cent excreted via the lungs, some 5 per cent of the injected dose is excreted in the urine during the first 14 days, and a negligible amount in the feces (12). An additional 2 per cent is present in the red blood cells as hemoglobin and will not be eliminated until the red cells are destroyed at the end of approximately 120 days in normals (8).

Analysis of C^{14} content of the tissues obtained at autopsy from Patient 3 shows that approximately 10 per cent of the injected activity was still present in the tissues 106 days after administration of the labeled glycine. If this remaining activity is eliminated from the body by the lungs by a single first order rate process, the elimination half time for this component can be estimated by use of the equation $N = 1.44\ N_0\ T\frac{1}{2}$ where N (2.2×10^7 dis./min.) is equal to the total C^{14} to be excreted by this rate process and N_0 is the zero time extrapolated value of the component. N_0 can be estimated to be no greater than 120 dis./min. (0.05 dis./min./mg. $BaCO_3$) (2.4×10^3 mg. $BaCO_3$/min. breathing) since the effect of this component cannot be seen in the graph in Figure 3, which is typical of the four patients.

As calculated in this manner, the half time is approximately 90 days. This represents the minimum value that this component could take since what is probably the maximum value for the zero time extrapolated value of this component has been chosen.

Sprinson and Rittenberg (13) have calculated that there is a protein turnover component in man with a half time of 158 days. The data presented here suggest that there is a C^{14} turnover component associated with labeled glycine administration of at least 90 days, which is in fair coincidence with that of Sprinson and Rittenberg, considering the assumptions made.

Since approximately 90 per cent of the administered C^{14} is rapidly eliminated by processes which can at the present time be measured directly, the radiation dose from the small fraction which is retained for a longer period is negligible at the present administered dosage level of 100 microcuries, and thus there is no hazard involved in this use of C^{14}. Finally, it should be pointed out that even if there were no elimination of the administered C^{14}, 100 microcuries can still be considered a safe tracer dose since it will deliver on the average less than 0.006 r per day of radiation to the tissues.

SUMMARY AND CONCLUSIONS

1. The rate of excretion of C^{14} via the lungs when administered as Glycine-2-C^{14} in three patients has been presented.

2. This pulmonary excretion has been described in terms of three rate processes, the slowest of which has a half time of seven to 14 days.

3. In 55 days approximately 83 to 92 per cent of the C^{14} is excreted via the lungs, 5 per cent via the urine, and 2 per cent is retained in the red blood cells during their life span.

4. Autopsy data in Patient 3 suggests that there is at least a fourth component of breath excretion with a half time of 90 days or greater.

5. The lack of hazard involved in the use of C^{14} in the manner described is discussed from the standpoints of rapid excretion of 90 per cent of the dose and retention of approximately 10 per cent in chemical compounds having a longer turnover time.

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ADDENDUM

Patient 1 (*W. F.*) is a 29 year old white male who has had chronic lymphatic leukemia of three years duration

and who at the time of these studies was in almost complete clinical remission.

Patient 2 (*H. M.*) is a 52 year old white male with polycythemia vera of 12 years duration. At the time of these studies he was in hematological remission, but shortly thereafter began to relapse hematologically.

Patient 3 (*G. J.*) was a 52 year old white female with chronic myelogenous leukemia of one year's duration. At the time of these studies the white blood count was in the 300,000 range, the platelet count was 600,000 and the red blood count 3.0 and the hemoglobin 6.9 grams. This patient pursued, despite treatment, a down hill course and expired in August 1950.

Patient 4 (*M. D.*) is a 50 year old white female with polycythemia vera of 14 years duration. At the time of these studies the red blood count was 6.5, hemoglobin 15 grams, white blood count 6,500, platelets 280,000. The patient has maintained this hematological picture until the present time.

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EFFECT OF PLATELET EXTRACT ON HEMOPHILIC BLOOD

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The function of platelets in the clotting process is still a matter of controversy. In Morawitz's classic formulation (1) the role of furnishing thromboplastin was attributed to them. Brinkhous, who agrees with this concept, has demonstrated that platelet-free, "quasi-hemophilic" plasma does not clot (2). According to him (2, 3) the thromboplastin contained in platelets is set free during clotting through the action of a plasma factor functioning as a thrombocytolysin. The fundamental defect in hemophilia is thought to be a diminution or absence of this plasma factor. Other investigators (4, 5), however, have maintained that the quantities of thromboplastin found in platelets are too small to be of significance in coagulation. According to Quick (3, 6) they liberate an enzyme, thromboplastinogenase, which interacts with the plasma factor, thromboplastinogen, to form thromboplastin. Hemophilia again is interpreted to be a deficiency of the plasma factor, *i.e.*, a lack of substrate for the platelet enzyme. Ware and associates (7) analyzed platelet extracts and found traces of thromboplastin, an accelerator analogous to serum Ac-globulin and a factor which enhances the action of thrombin on fibrinogen.

During the study of two patients with hemophilia-like disease caused by circulating anticoagulants which seemed to inhibit either the formation or utilization of thromboplastin, we found (8) that a concentrated homogenate[3] of ground-up platelets was able to restore the markedly prolonged clotting times to normal. Quick and Stefanini (9) have suggested that in hemophilia-like disease the anticoagulant may inhibit the action of the platelet enzyme on the plasma thromboplastin precursor, and the acceleration of clotting observed by us seemed to be attributable to an overcoming or neutralization of the anti-platelet enzyme factor by the extract. However, we observed a similar shortening of the coagulation time when such a concentrated platelet preparation was added to hemophilic blood (8), which is deficient in plasma factor but has normal platelets. It was established that these extracts had definite thromboplastic activity, and the problem presented itself whether this could account for the noted effects. It was decided, therefore, to investigate and compare the action of platelet homogenates and dilutions of tissue thromboplastin on the clotting time and prothrombin consumption time of a group of patients with hemophilia.

METHODS

1) *Preparation of Platelet Extract.*

A modification of the procedure described by Mann and colleagues (10) was employed. Citrated blood (one part of 1.34% sodium citrate to four parts of whole blood) is collected with an ordinary syringe and centrifuged soon after collection at 700–800 RPM for one half hour. The supernatant platelet-rich plasma is respun at the same speed for 15 minutes, removed from the few remaining red cells, and again centrifuged in Wassermann tubes at 3,000 RPM for 30 minutes.

The platelets, which still may be contaminated with a few red and white cells, are found to form a sticky pellet at the bottom of the tubes. The supernatant plasma is poured off and measured; the pellet is dislodged and broken up with a wooden applicator and shaken vigorously with a saline-citrate solution (0.1 cc. of sodium citrate to 10 cc. of normal saline). The platelets are then sedimented again by centrifuging at 3,000 RPM for 10 minutes. This process of washing is repeated three more times. After the final washing the platelets are suspended in saline-citrate and triturated in a mortar for two to three minutes. The final volume of this suspension of ground-up platelets in saline-citrate is adjusted so that 1 cc. of platelet homogenate corresponds to 10 cc. of original plasma, care being taken to correct for the 20% dilution of the plasma with citrate. The platelet extract is then ready for use but can be kept in the frozen state for 24 hours without loss of potency.

[1] Ruth Reader Fellow in Hematology.

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[3] For the sake of convenience the terms platelet "extract" and "homogenate" will be used interchangeably.

2) Prothrombin Time.

Determinations were made with the one-stage method of Quick, using a commercial preparation of thromboplastin (Maltine). Normal values range from 11 to 14 seconds.

3) Clotting Time.

The Lee-White clotting time was done always in three test tubes (8 × 75 mm.) at room temperature. Values up to 20 minutes are considered to be normal (11). When the effect of various substances on the clotting time was tested, the material was placed first into each tube, 1 cc. of blood then added, the tube inverted once, and the clotting time measured in the usual manner.

4) Prothrombin Consumption Time.

This test was performed by a slight modification of Quick's (4) method. About 5 cc. of blood were drawn into an ordinary syringe with a silicone-coated needle by clean venipuncture and discarded. Then a blood sample for the test was collected in an iced, siliconized syringe through the same needle and centrifuged in iced, siliconized tubes at 1,700 RPM for 10 minutes. The plasma was tranferred in 1 cc. portions to uncoated tubes containing the substance whose effect on prothrombin consumption time was to be tested and allowed to clot at 37°C. One hour after coagulation the clot was rimmed and the tube centrifuged at 4,500 RPM for one minute. The prothrombin consumption time was determined immediately using freshly prepared $BaSO_4$-plasma as a source of fibrinogen. Normal values, in our hands, are above 17 seconds, signifying that more than 60% of the prothrombin activity has disappeared. Further determinations of prothrombin consumption time were made on the serum, which had been allowed to remain in contact with the clot at 37°C, at varying intervals up to one and one half hours. There is normally a progressive lengthening of the prothrombin consumption time within this period.

5) Preparation of Thromboplastin Dilutions.

Thromboplastin (Maltine) was prepared as for the routine prothrombin time determination. The final thromboplastin solution was tested on normal oxalated plasma. When found to be satisfactory (prothrombin time of 11 to 14 seconds), the thromboplastin solution was diluted with normal saline until the desired concentrations were obtained.

RESULTS

1) Determination of the Thromboplastic Activity of Platelet Extracts.

The one-stage prothrombin time procedure of Quick was used as an index of the thromboplastic activity of platelet homogenates. 0.1 cc. of platelet extract and of increasing dilutions of thromboplastin were substituted for undiluted thromboplastin, and the "prothrombin times" determined on normal, oxalated plasmas in the usual manner. This comparison was performed separately for each platelet preparation, and their average thromboplastic activity was found to be equivalent to that of a thromboplastin dilution of approximately 1 : 800. Table I gives typical values obtained in such experiments with four different platelet extracts.

2) Effect of Platelet Extracts on Clotting Time.

Platelet extracts always were effective in shortening the clotting times of the bloods of eight hemophiliacs (Table II). Although the amount of extract used in these experiments was usually 0.3 cc., as little as 0.05 cc. was sufficient to bring the clotting times into the normal range in almost all instances. This effect cannot be attributed to contamination with either plasma or red cells, even though it has been shown that quantities of normal plasma as small as 0.001 cc., when added to 2 cc. of hemophilic blood, increase the rate of clotting considerably (12). When the clot-promoting ability of equal quantities of dilutions of fresh oxalated plasma, triturated washed red cells, and platelet extracts was compared, significant shortening of the clotting occurred only with the diluted plasma

TABLE I

Comparison of thromboplastic activity of platelet extracts and dilutions of thromboplastin on normal plasmas

Source of thromboplastin	"Prothrombin" time in seconds			
	Subject A	Subject B	Subject C	Subject D
Thromboplastin (Maltine) undiluted	12	13½	12	14
Thromboplastin (Maltine) 1: 500	63	64	61	66
Thromboplastin (Maltine) 1: 800	75	72	—	79
Thromboplastin (Maltine) 1:1,000	80	81	71	94
Thromboplastin (Maltine) 1:2,000	93	88	85	93
Platelet extracts	74	66	74	90

TABLE II

Effect of platelet extracts and of thromboplastin dilutions on clotting times of hemophilic blood

Patient no.	Effect of platelet extract (clotting times in minutes)		Effect of thromboplastin dilutions (clotting times in minutes)				
				Thromboplastin dilutions			
	Normal saline 0.3 cc.	Platelet extr. 0.3 cc.	Normal saline 0.3 cc.	1:500 0.3 cc.	1:800 0.3 cc.	1:1,000 0.3 cc.	1:2,000 0.3 cc.
1	26½	9	23	8	8	8	11
2	33	9	35	8	—	9	13
3	35	15	35	9	15	24	25
4	44	14	22	8	—	9	10
5	over 150	8	—	—	—	—	—
6	130	13	130	7	—	8	34
7	69	17	69	8	11	21	21
8	120	16	120	6	8	9	—

and the platelet extracts. The potency of the platelet preparations was essentially unaffected by repeated washings prior to trituration, demonstrating that contamination with antihemophilic globulin was not involved (Table III).

3) Effect of Thromboplastin Dilutions on Clotting Time.

Saline thromboplastin dilutions of 1 : 500 and 1 : 800 reduced the clotting times to normal values in all instances (Table II). Higher dilutions (1 : 1,000, 1 : 2,000) were less effective in some patients but always caused a significant shortening of the clotting time. A rough quantitative relationship seems to exist between the rate of clotting and the amount of thromboplastin added.

4) Effect of Platelet Extracts on Prothrombin Consumption Time.

The prothrombin consumption time was determined on 27 separate occasions in our eight patients. In five patients the values obtained never were higher than 13 seconds, whereas in three (cases 1, 3, and 4, Table IV) the prothrombin consumption time showed definite, spontaneous fluctuations, sometimes even reaching the normal range. In two instances we observed a normal prothrombin consumption time in the presence of a prolonged clotting time. The effect of platelet extracts was tested on 12 separate occasions (Table IV). A change to normal values—above 17 seconds—was obtained six times (twice with 0.2 cc., and four times with 0.4 cc.), whereas in five experiments the prothrombin consumption time was raised only slightly. The ability of equal amounts of platelet extract to produce an increased prothrombin consumption time varied in one patient (case 6) on different days. This cannot be blamed on differences in potency of the homogenates used, since extracts were found to be effective in one patient, but ineffective in another when tested simultaneously. Therefore, it seems

TABLE III

Comparison of the effect of platelet extract, triturated red cells, and diluted normal plasma on the clotting time of hemophilic blood

Material added (0.3 cc.) to 1 cc. of hemophilic blood	Clotting time in minutes			
	Subject A	Subject B	Subject C	Subject D
None	34	—	—	—
Normal saline	33	88	95	22½
Platelet extract, washed 3×	9	18	14	12
Platelet extract, washed 5×	—	18	17	13½
Platelet extract, washed 9×	—	21	25	12½
Triturated, washed red cells (1:10)	25	—	83	—
Diluted plasma (1:10)	24	16	14	—
Diluted plasma (1:100)	—	29	22	—

TABLE IV

Prothrombin consumption time one hour after clotting with addition of various materials to 1 cc. of "native" plasma

Patient no.	Prothrombin time in seconds	Prothrombin consumption time in seconds								
		Nothing added	Normal saline 0.2 cc.	Platelet extract		0.2 cc. Thromboplastin dilutions added				
				0.2 cc.	0.4 cc.	1:200	1:500	1:800	1:1,000	1:2,000
1	12½	15	—	—	—	—	—	—	—	—
	12	17	—	—	—	—	—	—	—	—
	12½	13	—	—	—	—	—	—	—	—
	—	11½	13	18	—	—	14½	13½	13½	13
2	13	11½	—	—	—	—	—	—	—	—
	14	13	—	—	—	—	—	—	—	—
	—	12	—	—	—	—	—	—	—	—
	—	12	12½	—	17	—	17½*	—	13*	—
3	13	22	—	—	—	—	—	—	—	—
	14	22½	—	—	—	—	—	—	—	—
	12½	11½	—	—	17½	—	—	—	—	—
	—	16	—	—	—	—	—	—	—	—
	13	13	—	—	—	—	—	—	—	—
	13	19†	—	—	—	—	—	—	—	—
4	11½	11½	—	—	—	—	—	—	—	—
	11½	12	—	—	17	—	—	—	—	—
	—	18‡	—	—	—	—	—	—	—	—
5	11	19½	—	—	—	—	—	—	—	—
	13	11	11½	15½	17	18	17	18½	16	—
	—	10	—	—	—	—	—	—	—	—
	—	12½	—	—	—	—	—	—	—	—
6	11½	10½	10½	23½	—	—	—	—	—	—
	—	10½	—	14	—	—	12	—	11	10½
	11½	9½	9½	13½	14½	14	11	10½	11	—
7	13½	no clot	9	13½	15	—	10½	10	10½	11
	—	no clot	—	16	—	—	—	—	—	—
8	14	no clot	11	14½	15½	—	12	15½	15½	11½
	13½	10	10	15	16	23	20½	10½	—	—

* Only 0.5 cc. of plasma used
† Clotting time 35 minutes
‡ Clotting time 22 minutes

likely that the irregular responses are determined by variables present in the plasmas.

5) Effect of Thromboplastin Dilutions on the Prothrombin Consumption Time.

Thromboplastin in dilutions of 1:1,000 or 1:2,000, added to hemophilic plasma, had little effect. Larger concentrations (1:200 to 1:800), however, were capable of producing a normal prothrombin consumption time at times. No parallelism appeared to exist between the ability of dilute solutions of thromboplastin to shorten the coagulation time and to increase the prothrombin consumption time. A quantity of thromboplastin, which clots hemophilic blood with normal speed, may be unable to affect the prothrombin consumption time in the same patient. These results are to be expected, since it is known that the prothrombin consumption time of hemophilic blood—which has clotted because of contamination with tissue thromboplastin during venipuncture—may be pathologic in the presence of a normal clotting time (13). Again it is to be noted that individual patients showed marked differences in the extent to which thromboplastin dilutions could alter their prothrombin consumption time.

DISCUSSION

Our experiments showed that concentrated aqueous homogenates of human platelets con-

sistently reduced the clotting times to normal in eight hemophilic subjects. The effect on the prothrombin consumption time, however, was not always significant. Platelet extract was found to possess a thromboplastic activity equivalent to that of a dilution of lung thromboplastin of about 1 : 800 as measured with the one-stage "prothrombin time" technique. Thromboplastin solutions of this potency exerted approximately the same effect on the clotting and prothrombin consumption times as the platelet preparations. These findings seem to indicate that the platelet extracts most likely acted by virtue of their thromboplastic activity. We choose this term since the nature of "thromboplastin" is not clearly defined at present. Many materials exhibit thromboplastic activity, but the responsible agents are not necessarily identical. At least two thromboplastins with different chemical properties have been found in lung and brain tissues, respectively (14).

Previous investigations have demonstrated that platelets contain traces of thromboplastin (4, 5, 7, 15), an accelerator analogous to serum Ac-globulin (7, 15), and a factor enhancing the action of thrombin on fibrinogen (7). It is unlikely that the platelet accelerator accounts for the observed results, since it has been shown that purified Ac-globulin does not influence significantly the clotting time of hemophilic blood (16). A potentiation of the action of thrombin on fibrinogen does not explain the increased prothrombin consumption time which was observed on several occasions and, therefore, cannot be the primary mechanism by which the platelet homogenate exerts its effect.

Other clotting factors also can be ruled out. Thrombin is not present in the platelet homogenates since they do not clot $BaSO_4$-plasma (17). No serum prothrombin conversion accelerator has been found in platelet extracts (18). Plasma antihemophilic globulin is removed by repeated washings during the preparation of the platelet extracts. It does not appear reasonable that thromboplastinogenase, the platelet enzyme specifically postulated to interact with the plasma factor thromboplastinogen (antihemophilic globulin), should have a direct activating effect on prothrombin. This would have to be assumed since no, or very little, thromboplastinogen can be considered to be present in hemophilic blood. By exclusion, therefore, it appears that platelet extract acted by virtue of its thromboplastic activity. Our comparative studies with dilutions of tissue thromboplastin, while not furnishing direct evidence, also strengthen this interpretation by demonstrating that only very small amounts of thromboplastin are fully capable of producing all the effects noticed with platelet extract.

Most investigators agree that interaction of both platelet and plasma factors is necessary to form an active thromboplastin for normal clotting. Conley and co-workers (19) have shown that "complete" clotting—as measured by normal clotting and normal prothrombin consumption times—takes place only when both factors are present. In hemophilia the plasma factor is absent or decreased, while the platelets now are considered to be normal (2–4); in severe thrombocytopenia the picture is reversed (20). In both conditions the prothrombin consumption time is usually abnormal; in hemophilia the clotting time is increased, but in thrombocytopenia it is within normal limits. In the latter disorder this finding is ascribed to either release of sufficient thromboplastinogenase by the few remaining platelets (3, 4) or direct activation of the plasma thromboplastin precursor by glass surfaces (21). In our experiments platelet extracts on several occasions produced both normal clotting and prothrombin consumption times in hemophilic subjects in whom the plasma factor was absent or markedly decreased. Since the extract also was free of this latter factor, it must have provided in these instances the end product of the interaction of platelet and plasma factors, *i.e.* thromboplastic activity.

Patek and Stetson (22), on adding a suspension of platelets of the same concentration as that found in normal plasma, observed no effect on the clotting time of hemophilic blood. This apparent discrepancy with our data may be explained by considering that the platelet preparations used in our experiments were *1*) triturated and *2*) 200 times more concentrated than theirs. It does not seem permissible to apply directly our interpretation of the effect of the extracts to the function of platelets *in vivo*.

Most of our data show a lack of parallelism between the changes in the clotting and the prothrombin consumption times, regardless of whether tissue thromboplastin or platelet homogenate was the agent studied. In other words, while either

reagent uniformly accelerated clotting, the "consumption of prothrombin" often was not increased significantly. Our data also suggest that patients with hemophilia show definite fluctuations in their clotting status. This is indicated by spontaneous variations in their clotting and prothrombin consumption times when tested repeatedly over prolonged periods in the absence of hemorrhage or treatment with transfusions. It is well known that hemophiliacs may exhibit periods of quiescence of bleeding manifestations to be followed by intervals marked by hemorrhages of varying severity (23). If it is assumed that these clinical and laboratory observations reflect differences in the patient's ability to form thromboplastin, this mechanism can be invoked to offer an explanation for the apparent inconsistencies of our observations. Both platelet extract and dilutions of thromboplastin supply thromboplastin in addition to that produced by the patient himself. It depends on the patient's own thromboplastin production—which does not seem to be constant—whether this extraneously furnished thromboplastin is sufficient to give a normal "consumption of prothrombin." Likewise, the occasional normal values of the prothrombin consumption time, which have been observed also by others (24), could be a reflection of a temporary improvement in the patient's clotting status. It is to be emphasized, therefore, that a normal prothrombin consumption time, even in the absence of therapy, does not exclude the diagnosis of hemophilia.

Considerable difference of opinion exists about what actually is being measured when the "prothrombin consumption time" is determined. It has been stated (18, 25) that during coagulation besides the disappearance of prothrombin, which lengthens the prothrombin time of the serum, accelerator factor(s) evolve, which shorten it. The release of adsorbed thrombin from the fibrin meshwork also may influence this value (26). However, even when these mechanisms are taken into consideration we are at a loss to explain our two observations of a normal prothrombin consumption time in the presence of a prolonged coagulation time.

SUMMARY

1. Addition of a concentrated homogenate of ground-up platelets consistently reduces the clotting times of hemophilic bloods to normal. The prothrombin consumption time is increased regularly but not always significantly.

2. These effects can be explained most satisfactorily by attributing them to the thromboplastic activity of the platelet preparations.

3. When tested repeatedly over prolonged periods hemophiliacs, in the absence of therapy, may occasionally show a normal prothrombin consumption time. It is postulated that the fluctuations in the clotting status may reflect variations in the patient's ability to produce thromboplastin.

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PEPTIDASE ACTIVITY IN LEUCOCYTES, ERYTHROCYTES AND PLASMA OF YOUNG ADULT AND SENILE SUBJECTS [1, 2]

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INTRODUCTION AND REVIEW OF THE LITERATURE

In the present work an attempt was made to find out whether the peptidase activity of the human blood changes with age. Preliminary to this a study of the characteristics of peptide hydrolysis was undertaken, and the degree of enzymatic activity in the various blood constituents was investigated.

Grassmann and Heyde (1) demonstrated the ability of serum to hydrolyse di- and polypeptides. Various authors have found that the peptidase activity of serum is increased in certain pathological conditions, such as during inflammatory processes, after bone fracture, and after burns (1–4). Concerning the possible source of the serum peptidases Zamecnik, Stephenson, and Cope (4) suggest that they may derive from muscle, skin, subcutaneous tissue and erythrocytes. Fruton and co-workers (5, 6) believe that peptidases may have a common origin, most probably the lymphoid cells present in the various tissues, and that the peptidase activity of serum might be due to the disintegration of lymphoid tissue.

Peptidase activity in erythrocytes has been demonstrated by a number of investigators (4, 7–10). Johansen and Thygesen (8) showed that per unit volume the rate of hydrolysis of alanylglycylglycine (AGG) by human erythrocytes is 40–50 times higher than by serum. Merten and Winschuh (11) report an increase in di-peptidase activity of human erythrocytes in various pathological conditions.

It has long been known that leucocytes are able to break down peptides [compare the literature review of Husfeldt (10); Merten and Winschuh (11)]. Husfeldt (10) demonstrated di-, tri-, and tetra-peptide hydrolysis by leucocytes from a normal subject and a patient with myeloid leukemia. Hydrolysis of alanylglycine by leucocytes from patients with lymphatic leukemia has been observed by Oelkers (12).

Little is known about the relation of the age of the organism to the proteolytic activity of blood. Goldenberg and Kondrachina (13) reported that the rate of autolysis of rabbit serum at pH 1 decreases with the age of the animal. Maver and co-workers (14), on the other hand, noted that serum from old normal rats apparently hydrolysed dl-leucylglycine at a faster rate than did the serum from younger rats.

SUBJECTS

Two groups of subjects were studied. The first group included 15 members of the staff, nine male and six female, ranging in age from 19 to 32 years. The second group of 12 comprised a number of senile patients, five male and seven female, in a mental institute. The members of this group were not bedridden, and none of them suffered from any known organic or infectious disease. Their ages ranged from 67 to 90 years.

METHOD

Twenty ml. of non-fasting blood was obtained from the cubital vein in the morning, and was poured immediately into flasks containing 40 mg. of potassium oxalate. A white blood count and differential count were made immediately. The white blood cells were separated by centrifugation of whole blood in waisted tubes (15). Plasma was removed by means of a capillary pipette. The buffy layer which contained white blood cells and platelets, plus a few red blood cells, was transferred with a second capillary pipette to a centrifuge tube, suspended in about 2 ml. of physiological saline solution and centrifuged at low speed for approximately two minutes. (Longer centrifugation caused the cells to adhere together in small clumps which could not be broken up on shaking.) After centrifugation the clear supernatant was sucked off with a pipette and the cells resuspended in from 2 to 4 ml. of saline and stored overnight in the refrigerator. The red blood cells were pipetted with a long capillary pipette from the bulb of the centrifuge tube and washed in the same manner as the white cells. The concentration of cells in these saline suspensions was determined by means of the standard

[1] This research was aided by a grant from the National Research Council of Canada.

[2] An abstract of this paper appeared in Proceedings of the Canadian Physiological Society, October, 1950.

clinical procedures, using Turck's and Hayem's dilution fluids. All counts were made in quadruplicate.

The substrate used was glycylglycylglycine (GGG). Hydrolysis was measured by the micro-titration technique of Grassmann and Heyde. The reaction mixture was set up in triplicate in glass-stoppered 2 ml. volumetric flasks. It was composed of 1 ml. of 0.1 M substrate, 0.2 ml. of 0.01 M cobalt sulfate, 0.01 ml. toluene and 0.8 ml. of plasma, or 0.6 ml. of distilled water and 0.2 ml. of cell suspension containing approximately 10^8 leucocytes or 10^8 erythrocytes.[3] The reaction was carried out in a water bath at a temperature of 39°C and at a pH of 7.0, maintained within less than 0.1 unit by the buffering effect of the substrate which had been brought to pH 7.0 with 0.5 N NaOH. Hydrolysis during a two to four hour period was measured at frequent intervals by titrating 0.2 ml. samples with 0.01 N KOH (final concentration 90% alcohol), using thymolphthalein as indicator. At the dilution of red blood cells used no serious difficulty with the endpoint was encountered.

RESULTS

Preliminary experiments regarding some of the characteristics of the hydrolysis of GGG will be described first. The rate of hydrolysis by plasma, white cells, or red cells, is constant until about 70% of the substrate is split, assuming complete splitting of one peptide linkage (see Figure 1). Beyond 100% theoretical splitting of a single bond of the tripeptide, hydrolysis continues but at a slower rate. To what extent the breakdown of the di-peptide contributes to the earlier stages of GGG hydrolysis is not known. The rate of hydrolysis by plasma, erythrocytes, and leucocytes was found

TABLE I

The effect of dilution of blood components on the rate of hydrolysis of GGG

Substrate 0.05 M GGG, pH 7.0

Enzyme source	Concentration per ml.	Per cent hydrolysis per hour	
		Observed	Calculated for 0.2 ml./ml.
Plasma*	0.10 ml.	7.0	14.0
	0.15 ml.	9.5	12.7
	0.20 ml.	12.0	12.0
	0.26 ml.	17.0	12.6
	0.40 ml.	24.4	12.2
		Observed	Calculated for 1 million cells/ml.
R.B.C.	96 million cells	45	0.48
	72 million cells	34	0.47
	48 million cells	23	0.48
W.B.C.	1.34 million cells	16.0	11.2†
	0.96 million cells	12.7	12.3†

* The plasma was slightly hemolysed which accounts for the high rate of hydrolysis.

† Values corrected by subtraction of the rate of hydrolysis due to the presence of erythrocytes.

[3] In order to facilitate cytolysis the cell suspensions were incubated with the distilled water and the cobalt sulfate solution for 15 minutes before the addition of substrate, and the tubes vigorously agitated. Cell counts immediately after substrate addition (zero experimental time) and during the course of hydrolysis showed that the red cells were completely hemolyzed but only about 40% of the white cells from both young adult and senile subjects were cytolyzed at zero time and about 50% after two hours incubation. In all experiments the peptidase activity is based on the cell count before cytolysis occurred.

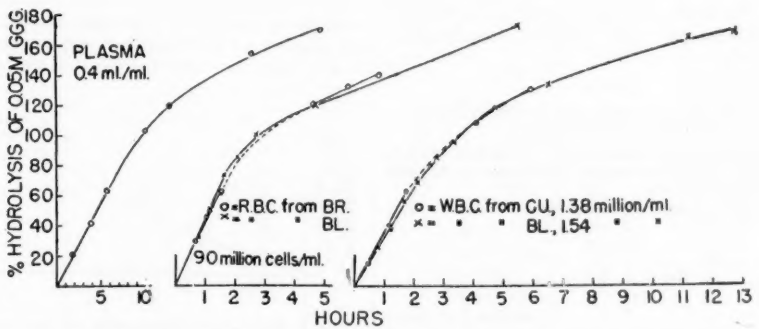

FIG. 1. THE COURSE OF HYDROLYSIS OF GGG BY PLASMA, LEUCOCYTES, AND ERYTHROCYTES

The reaction follows a similar pattern with all three enzyme sources, *i.e.*, the hydrolysis rate is constant until about 70% of the substrate is split; after 100% theoretical splitting of one peptide bond of the tripeptide further hydrolysis occurs but at a slower rate.

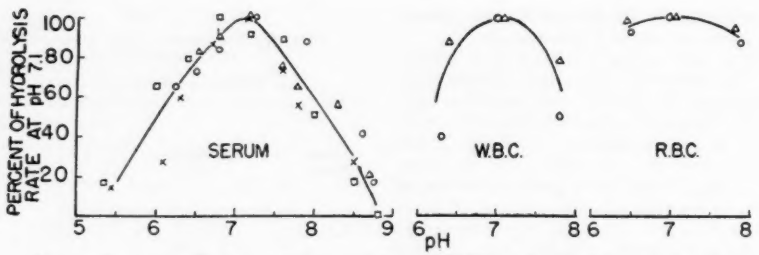

FIG. 2. EFFECT OF pH ON THE HYDROLYSIS RATE OF GGG BY SERUM, LEUCOCYTES, AND ERYTHROCYTES

The substrate was adjusted to the desired pH by adding the required amounts of HCl or NaOH. The drift in pH during the course of hydrolysis was negligible. Similarly marked points in each curve were obtained on samples of the same blood.

to be directly proportional to the concentration of these constituents in the reaction mixture (see Table I). The effect of metal ions on the hydrolysis rate is shown in Table II. Cobalt activates, magnesium and manganese inhibit the hydrolysis by plasma, red cells and white cells. It may be noted that a similar effect of these metal ions has been observed in this laboratory with the hydrolysis of l-leucylglycylglycine (LGG) by human serum (2). The effect of pH on the hydrolysis of GGG by various blood constituents is shown in Figure 2. Optimal hydrolysis occurs around pH 7.1.

The similar characteristics of the hydrolysis of GGG makes it likely that the same enzyme mechanism is involved in plasma, leucocytes, and erythrocytes.

The effect of temperature was studied using serum as the source of the enzyme. The rate of hydrolysis increases with increasing temperature between 0° and 48°C, but at higher temperatures inactivation sets in (see Figure 3).

Regarding the relative activity of the blood components, it may be seen from Table III that in normal young people the average rate of hydrolysis per cell is about 40 times higher in white blood cells [4] than in erythrocytes. In view of the high concentration of erythrocytes in blood, however, the activity of a sample of whole blood is mainly due to the hydrolysis by red blood cells. Using the average rates of hydrolysis of GGG in young adults and average normal values for red and white cell counts and plasma volume (16) it may be estimated that in a unit of whole blood the activity of the white blood cells is about six times greater, that of the red blood cells about 100 times greater than that of plasma. If the activity

TABLE II

Effect of metal ions on the hydrolysis of GGG by plasma, leucocytes, and erythrocytes

Per cent hydrolysis per hour of 0.05 M GGG of pH 7.0, calculated for a concentration of 0.2 ml. plasma, or 10^8 cells per ml. reaction mixture.

	Plasma	R. B. C.	W. B. C.	
			Age of subject	
			28 Years	67 Years
No activator	4.0	0.25	15	21
Co (0.001 M)	5.6	0.43	19	31
Mn (0.001 M)	3.1	0.16	10	16
Mg (0.01 M)	3.5	0.14	9	13

[4] The hydrolysis of GGG by white blood cells has been corrected by subtraction of the rate of hydrolysis due to the presence of erythrocytes in the reaction mixture. In most cases this value was less than 10% of the total rate.

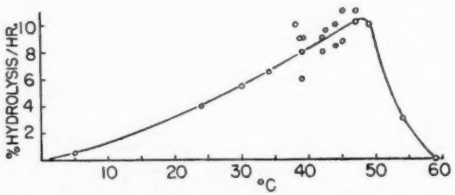

FIG. 3. EFFECT OF TEMPERATURE ON THE HYDROLYSIS RATE OF GGG BY SERUM

Values represent per cent hydrolysis by 0.2 ml. of serum per ml. reaction mixture. Serum was obtained at various times from one subject.

of one volume of plasma is compared with that of a volume of packed white and packed red cells, ratios of 1 to 480 to 125 are obtained. A similar proportion of enzyme activity among blood components was observed in experiments in which dl-leucylglycylglycine was used as substrate. The relative rates for plasma and red blood cells are somewhat higher, but of the same order of magnitude as the comparative values for AGG hydrolysis by human serum and erythrocytes estimated by Johansen and Thygesen (9). No clear cut data in the literature on the relative activity of white blood cells are known to us.

From Table III it may be seen that the rate of hydrolysis of GGG by white blood cells is considerably higher in the old age group than in the young adult group. The average values of hydrolysis of 0.05 M GGG per hour for one million cells per ml. of reaction mixture are 26 and 15% respectively. The difference is statistically significant ($P < 0.001$). (The averages for male and female subjects correspond closely in both groups.) When the values for plasma or red blood cells are compared no significant difference between the two groups is seen. No correlation between activity of white blood cells and the percentage of

TABLE III

Hydrolysis of GGG by leucocytes, erythrocytes, and plasma of young and old people

Name	Age	Sex	Total W.B.C.	% Poly.	% Lym.	% Stab.	Hydrolysis % per hour*			Remarks
							10^6 WBC/ml. reaction mixture	10^6 RBC/ml. reaction mixture	0.2 ml. plasma/ml. reaction mixture	
VE	27	M		17	47	26	18	0.35		
WO	22	M		26	67.5	0.5	17	0.27		
CH	25	M		56	42	0	14	0.27		
			6,900	59	35	4	15	0.41	5.5	
WI	21	F		69	25	0	14	0.32		
CA	23	M		61	33	0	18	0.25		
BR	23	M	9,700	34.5	39	19	13	0.26		
			8,800	50	41	6	12	0.34	7.0	Repeat 2 weeks later
CU	28	F	8,250	52	41	0	11	0.41		
			11,675	51	39	4	20	0.36		1st day menstruation
			21,800	54	33	8	15	0.46		2nd day menstruation
DY	22	F	6,950	51	40	1	18	0.38		
PU	22	F	5,950	57	34	5	16	0.39	6.2	
KO	26	F	7,500				9	0.44	6.5	
DE	28	F					16	0.41		Jan. 6, 1950
				65	27	6.5	19	0.43		April 4, 1950
							21	0.46		May 15, 1950
HO	26	M	7,100				14	0.46		
MA	32	M					20	0.42		
KA	19	M	11,300	56	38	0	10	0.40	6.8	
EL	26	M	4,800				16	0.47		
Average							15	0.38	6.4	
DA	76	F		70	14	13	28	0.33		
			13,450	81	7	7.5	22	0.34		Repeat 13 days later
SM	68	F		47	48	0	18	0.31	7.5	
MI	82	M					26	0.45		
			6,900	53	40	4	28	0.41	7.8	
HA	90	M	9,000	41	53	4	26	0.35	7.0	
			6,550				26	0.31	7.2	Repeat 2 months later
KY	94	F	11,050	60	32	4	30	0.48	5.5	
CA	86	M	9,750	61	32	4	32	0.43		
NO	84	M	10,200	40	57	1	20	0.50	7.8	
McG	87	F	10,775	58	34	2	30	0.47		
HE	89	F	10,525	59	38	2	33	0 38		
ME	86	F	8,250				26	0.24	6.2	
BA	67	F					25	0.40		
BR	71	M		42.5	48.5	8.5	23	0.35		
Average							26	0.39	7.0	

* Per cent per hour of the hydrolysis expected on the complete splitting of one peptide linkage.

polymorphonuclear leucocytes and lymphocytes in the differential count was apparent, and the slight difference in the distribution of the cell types among the two groups could not account for the difference in enzymatic activity. The response to metal ion activation was compared in two subjects (Table II) and no significant difference was observed. The possibility that the difference in rate among the two groups is due to an increased state of metal activation inherent in the white blood cells of the old age group is therefore unlikely. Although no pH optimum for hydrolysis of white blood cells from old people was determined, it is evident that the differences in activity cannot be due to a different response to pH since all studies were carried out in the optimum pH range for the activity of white blood cells in young people.

DISCUSSION

The increase in peptidase activity of white blood cells from aged subjects is noteworthy from a clinical standpoint in view of the role of leucocytes in processes of infection and tissue repair. It is possible, however, that the rise in peptidase activity might be a manifestation of increased protein metabolism in general, associated with old age. It must be pointed out that the old age group was composed largely of subjects who were institutionalized because of senile mental changes, and whose food intake was probably lower than that of the control group. The study will be continued on mentally normal old people. It is of interest that insulin, a polypeptide, has been found to lose its blood sugar reducing power when it is incubated with whole blood, and that blood from old people inactivates the hormone at a significantly higher rate than blood from young people (17). There was an indication that the leucocytes were responsible for this effect. This would be in line with the present observations, provided that a hydrolytic mechanism is involved in the inactivation of insulin.

The rise in peptidase activity of the white blood cells in the senile group was not accompanied by a significant rise in peptidase activity of plasma. This does not necessarily exclude the possibility that white blood cells are a source of plasma peptidase since the rate of dissolution of leucocytes rather than the actual activity of living cells may determine the level of enzymes in plasma. It has been observed that the leucocytes circulating in the blood stream of old people are older cells than those in the blood from young persons (18). This indicates that the rate of turnover is lower in old age. It should, however, be kept in mind that per unit of whole blood by far the greatest enzyme activity is found in the red blood cells. Whether erythrocytes, leucocytes, platelets, or other tissue cells are the main source of the enzyme in plasma can only be determined if the rate of breakdown and turnover, as well as the relative enzyme activity of these constituents, is known.

SUMMARY

1. The hydrolysis of glycylglycylglycine (GGG) by the components of blood from young adult and from senile human subjects, and the effects of metal ions, pH, and temperature on the hydrolysis were studied.

2. Hydrolysis by plasma, erythrocytes and leucocytes was activated by cobalt, inhibited by magnesium and manganese and optimal at pH 7.1. In view of the similar characteristics of hydrolysis by the blood components it was concluded that the same enzyme mechanism must be involved in all cases.

3. The enzyme activity in the formed elements by far exceeded that of serum or plasma. In normal young people a volume of packed red cells was estimated to be over 100 times as active as a volume of plasma, and packed white blood cells nearly 500 times as active as plasma. Per unit cell the activity of leucocytes from young people was found to be about 40 times greater than that of the erythrocytes.

4. The rate of hydrolysis of GGG by leucocytes was found to be significantly higher in senile people than in young people, the average increase in rate being about 70%. On the other hand, no significant difference in rate of hydrolysis by plasma or erythrocytes of the two groups was observed.

5. The significance of these findings is discussed.

The authors are indebted to Miss Marylee Putnam for technical assistance.

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THE EFFECT OF BILATERAL STELLECTOMY UPON THE CEREBRAL CIRCULATION OF MAN [1]

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The role of the sympathetic nervous system in the intrinsic control of the cerebral circulation has always been controversial. Experiments heretofore have centered on the effects of stimulation of the cervical sympathetic nerves on changes in cerebral blood flow detected by instruments applied directly to the brain of animals. The results have generally agreed that cervical sympathetic stimulation causes only mild cerebral vasoconstriction. The marked effects of chemical agents (CO_2 in particular) in affecting the cerebral circulation have led to the probably correct conclusion that the intrinsic control of the cerebral circulation in health is predominantly mediated via chemical agents in the blood and that neurogenic vasomotor control is at best only weakly effective (1). However, recent clinical reports (2–5), which we can confirm from personal experience, on the efficacy of temporary blockade of the stellate ganglia in ameliorating symptoms of cerebral embolism and thrombosis, presumably by relieving collateral vasospasm, have increased interest in the role of the sympathetic system in the intrinsic control of the disturbed cerebral circulation.

Harmel and associates (6) studied the effects of bilateral stellate ganglion block with procain on the cerebral circulation of 13 unanesthetized normotensive and hypertensive patients and failed to find any effect whatsoever. They utilized the recently introduced nitrous oxide technique for determination of the cerebral blood flow (7) in their studies. Scheinberg (8) has recently confirmed their work.

We have had the opportunity to study the cerebral circulation of seven patients before and after bilateral stellate ganglionectomy and our findings are the subject of this report.

[1] This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

METHODS

Patients selected for study were necessarily suffering from abnormalities for which it was hoped bilateral stellate ganglionectomy would be of benefit. Four patients (L. S., M. M., J. W. and W. N.) had postencephalitic Parkinson's disease; one (B. D.) was clinically diagnosed as advanced cerebral arteriosclerosis; one (R. H.) had hypertension and advanced cerebral atrophy (air-encephalogram); and one (A. A.) suffered with an old hemiplegia as a complication of essential hypertension.

Cerebral blood flow (CBF) was determined by the nitrous oxide technique (7). Mean arterial blood pressure (MABP) was measured from the femoral artery by a damped mercury manometer attached directly to a needle in the artery. Blood oxygen and carbon dioxide analyses were carried out by the manometric technique of Van Slyke and Neill (9). Blood pH measurements were made anaerobically at 37° C. using a glass electrode. Carbon dioxide tension in the blood (pCO_2) was calculated from the nomograms of Peters and Van Slyke (9). Cerebral vascular resistance (CVR) was calculated from the formula $CVR = \frac{MABP}{CBF}$ (7). Cerebral oxygen consumption (CMR) was determined by multiplying the CBF by the difference in oxygen content of the arterial and internal jugular blood.

Prior to operation a control cerebral blood flow measurement was made. At an interval of three to seven days following bilateral stellate ganglionectomy the studies were repeated. The patients were afebrile and received no premedication on either occasion. Earlier postoperative observations were precluded by the fact that general systemic changes (pulmonary effects and fever) would have obscured the results. This was unfortunate since it is well known that most vessels tend to regain a portion of their tone within several days after sympathectomy.

RESULTS

The most striking finding in the series as a whole was the significant fall of CVR following bilateral stellate ganglionectomy, from a mean value of 2.6 to 2.0 resistance units ($p < .02$). There was not so clear an increase in the mean CBF from 45.6 to 55.6 cc./100 g./min. ($p > .05$) and in the CMR from 2.49 to 2.90 cc./100 g./min. ($p < .9$). Average values for the mean arterial blood pressure,

pulse rate, cerebral respiratory quotient, jugular and arterial pH and CO_2 tensions were relatively unaffected by the operative procedure.

Both internal jugular and arterial oxygen content were significantly ($p < .02$) decreased (by 1.3 Vol. %) and the carbon dioxide content increased (3 Vol. %) following the bilateral removal of the stellate ganglia. These changes are probably due to a fall in the hematocrit postoperatively, and in one patient in whom the hematocrit was determined pre- and postoperatively such reduction did occur. The lowered hemoglobin content of the blood would result in reduction of the blood oxygen content. Blood with a lowered hematocrit would contain relatively greater amounts of plasma and since plasma does have a higher CO_2 content than red cells one would expect the CO_2 content of blood with a reduced hematocrit to be higher. However, since the CO_2 tension of the blood was unaltered by operation the increased CO_2 content of the blood could not be the critical factor in altering the cerebrovascular resistance postoperatively. Indeed, there is no correlation whatsoever between the changes in CVR with changes in any of the blood gas contents.

It is apparent that patients with initially slow CBF obtained the greatest reduction in CVR from the operation, and as a matter of fact an excellent correlation (Figure 1) exists between the preoperative CBF and amount of decrease in CVR ($r = .86$).

Almost as good correlation (Figure 2) exists between the preoperative CVR and the fall in CVR ($r = .75$).

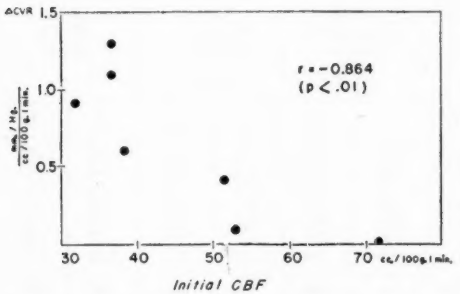

FIG. 1. RELATIONSHIP OF FALL IN CEREBROVASCULAR RESISTANCE FOLLOWING BILATERAL STELLECTOMY AND INITIAL CEREBRAL BLOOD FLOW

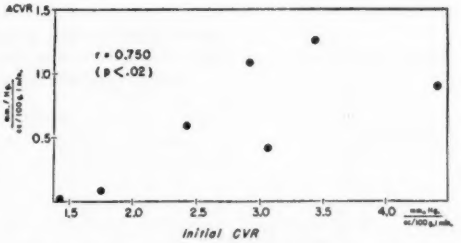

FIG. 2. RELATIONSHIP OF FALL IN CEREBROVASCULAR RESISTANCE FOLLOWING BILATERAL STELLECTOMY AND INITIAL CEREBROVASCULAR RESISTANCE

DISCUSSION

Our data confirm previous findings, chiefly in animals, that under normal conditions the intrinsic tone of the cerebral vessels is not mediated via the autonomic nervous system fibers passing through the stellate ganglia. However, the clear effect of stellectomy in reducing the CVR in patients with abnormally slow CBF and increased CVR would indicate that, in pathologic states with increased CVR, at least a portion of this increased resistance can be due to spasm mediated over sympathetic reflexes.

The element of spasm maintained reflexly can evidently be considerably greater when induced by physiological means in the intact human than when produced in experimental animals by external, even though direct, stimulation of the sympathetic nerves.

The question whether the blood in the internal jugular bulb is significantly contaminated with drainage from the face and the scalp after stellate ganglionectomy and thereby influences our calculations of the cerebral blood flow and other cerebral circulatory functions is a pertinent one. Observations which demonstrated a very minor contamination of internal jugular bulb blood by extracerebral drainage were made on intact individuals (10). However, one would expect, if this error were introduced by the procedure of stellectomy, that the arteriovenous oxygen differences and respiratory quotients would be materially altered after operation. This in fact did not occur.

The discrepancy of our results with those reported by Harmel, Hafkenscheil, Austin, Crumpton and Kety (6) probably is due to the fact that

TABLE I

Effect of bilateral stellate ganglionectomy on blood gas constituents

Patient	Age		Blood CO_2 content (*Vol. %*)				Blood CO_2 tension (*mm. Hg*)				Blood O_2 content (*Vol. %*)				Blood pH			
			Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular		Arterial		Int. jugular	
			I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
L. S.	38	Post-enceph. Parkinsonism	48.4	51.7	53.5	55.4	41	41	48	44	17.3	15.4	11.8	10.8	7.38	7.41	7.33	7.38
M. M.	51	Post-enceph. Parkinsonism	49.9	57.2	55.0	61.3	45	45	53	51	14.3	12.4	9.0	8.2	7.34	7.40	7.29	7.35
J. W.	27	Post-enceph. Parkinsonism	48.3	51.8	52.8	55.3	38	42	46	47	18.2	15.9	12.8	12.1	7.41	7.39	7.35	7.35
W. N.	42	Post-enceph. Parkinsonism	50.3	53.2	54.5	58.7	—	—	—	—	17.7	15.9	13.0	10.3	—	—	—	—
A. A.	43	Essential hypertension	51.0	53.8	55.9	59.6	—	—	—	—	14.0	13.1	8.8	7.0	—	—	—	—
R. H.	51	Cerebral atrophy	47.6	47.3	52.5	54.5	38	38	44	46	20.4	19.9	14.2	11.9	7.44	7.42	7.38	7.37
B. D.	59	Cerebral arteriosclerosis	50.2	51.1	55.6	56.5	44	44	52	50	16.5	15.3	11.1	9.6	7.37	7.38	7.31	7.34
Mean	44		49.4	52.3*	54.3	57.3*	41	42	49	48	16.9	15.4*	11.2	10.0*	7.39	7.40	7.34	7.36

I—Preoperative study
II—Post-bilateral stellate ganglionectomy study
* Denotes significant difference ($p < .02$)

the latter observers did not study patients with an initially reduced cerebrovascular resistance and cerebral blood flow. Scheinberg (8) did study such patients but performed only unilateral stellate blocks. Our results are based upon bilateral removal of the stellate ganglion.

We did not have the opportunity to study patients with acute cerebrovascular accidents. However, the marked reflex cerebral vasoconstriction demonstrated in some of our patients makes the existence of such a mechanism plausible in some cases of apoplexy and would explain the beneficial effects reported from stellate ganglia block in such instances. It also provides a rationale for cervical sympathectomy at the time of carotid ligation to overcome vasospasm that might result from reflex mechanisms brought into play, in some patients, by the ligation.

TABLE II

Effect of bilateral stellectomy on cerebral circulatory functions

Patient	(CBF) Cerebral blood flow (*cc./100 g./min.*)		(CMRO$_2$) Cerebral O_2 consumption (*cc./100 g./min.*)		(CVR) Cerebral vascular resistance $\frac{(mm.\ Hg)}{(cc./100\ g./min.)}$		(MABP) Mean arterial blood pressure (*mm. Hg*)		(A–V O_2) Arterio-ven. oxygen difference (*Vol. %*)		(R.Q.) Cerebral respiratory quotient	
	I	II	I	II	I	II	I	II	I	II	I	II
L. S.	37	50	2.0	2.3	3.2	1.9	119	95	5.5	4.6	.93	.80
M. M.	37	69	2.0	2.9	2.7	1.6	100	109	5.3	4.2	.96	.98
J. W.	71	68	3.8	2.6	1.4	1.4	99	96	5.4	3.8	.86	.92
W. N.	39	54	1.8	3.0	2.2	1.6	87	88	4.7	5.6	.90	.98
A. A.	51	55	2.7	3.4	2.8	2.4	141	132	5.2	6.1	.94	.95
R. H.	31	38	1.9	3.0	4.2	3.3	131	126	6.2	8.0	.79	.90
B. D.	53	55	2.9	3.1	1.8	1.7	98	94	5.4	5.7	1.00	.95
Mean	45.6	55.6	2.44	2.90	2.61	1.99*	111	106	5.4	5.4	.91	.93

I—Preoperative study
II—Post-bilateral stellate ganglionectomy study
* Denotes significant difference ($p < .02$)

SUMMARY

The cerebral circulation was studied in a series of seven patients before and after bilateral stellate ganglionectomy. A significant decrease in cerebrovascular resistance was found to result from stellate ganglionectomy. This decrease in cerebrovascular resistance following operation appears to be greatest in those patients with the initially slowest cerebral blood flow and initially most marked increases in cerebrovascular resistance.

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THE EFFECT OF VENOUS AND ARTERIAL OCCLUSION, AND SYMPATHETIC NERVE TONE, ON DIGITAL BLOOD FLOW [1]

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The normal range described originally (1) for blood flow in the fingertip, measured calorimetrically after vasodilatation by indirect heating, has been confirmed by Capriglione and Duque (2). Corrections for specific heat, gravity and temperature of blood (3), and the reinforcement of the effect of indirect heating by the intravenous administration of TEAC, subsequently raised the level of both normal and abnormal readings slightly. The normal range for the fingertip now is 0.25 to 0.38 cc./sq.cm. of skin/min., and for the toe 0.15 to 0.29 cc./sq.cm./min. (4). Venous obstruction plethysmography of the fingertip gives a normal range after indirect heating which is somewhat wider [6 to 12 cc./10 cc. of finger volume/min. (5)]. What is more, we have often observed considerable differences between single digital plethysmographic and digital calorimetric readings in the same subject under presumably similar conditions. Such discrepancies have also been noted between calorimetric and plethysmographic studies of the hand (6) especially at intermediate rates of flow, although the differences are ascribed to the "smoothing out" by the calorimeter of rapid fluctuations in flow recorded plethysmographically.

Hoobler, Avera, Little, Peet, and Bassett (7), employing the foot plethysmograph, have described considerably greater increases in blood flow during caudal anesthesia or after sympathectomy than after indirect heating, or after the intravenous injection of TEAC. These differences were attributed to the failure of indirect heat or TEAC to release sympathetic nerve tone completely. Arnott and Macfie (8), however, measuring blood flow calorimetrically, found that blocking the ulnar nerve with procaine did not increase flow in the fifth finger if sympathetic nerve tone had been previously released by indirect heat.

In an attempt to reconcile some of these discrepancies, the calorimetric method was subjected to exhaustive critical evaluation, the results of which are reported elsewhere (3). The assumptions involved in the method were sustained. It therefore seemed necessary to investigate some of the assumptions inherent in the plethysmographic method (9), the most vulnerable of these being the assumption that venous obstruction traps all the blood in the part. It also became necessary to investigate the completeness of release of sympathetic nerve tone by indirect heating supplemented by TEAC in the skin of the great toe.

METHODS

Accordingly, a series of experiments were set up wherein blood flow was measured calorimetrically after arterial and after venous obstruction, using heat supplemented by TEAC to release sympathetic nerve tone. Patients without vascular disease, and some with mild residua of "trench foot," were used as subjects. Heat was administered to the trunk by a cradle baker for from 30 to 45 minutes until positive heat balance was attained as manifested by generalized sweating. Then, TEAC (5 mg./Kg) was injected intravenously. Flow studies were begun approximately 10 minutes after the injection. For the great toe the compressing cuff was at the ankle, whereas for the fingertip (third or fourth) a Gaertner capsule was used at the proximal phalangeal joint. The arterial obstruction pressure varied between 180 and 220 and the venous obstruction pressure between 40 and 50 mm. Hg. Only those results were tabulated in which the flow within a minute after obstruction was at or near the original level. In approximately 10 per cent of subjects, venous obstruction produced a decrease in flow, which persisted long after release of pressure, indicating reflex vasoconstriction (see Figure 1). That psychic factors can "break through" indirect heat and less commonly through indirect heat supplemented by TEAC to produce reflex vasoconstriction is well known (10) and is demonstrated in Figure 2.

In addition, blood flow in the great toe measured calorimetrically after indirect heating and TEAC was com-

[1] Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or the policy of the Veterans Administration.

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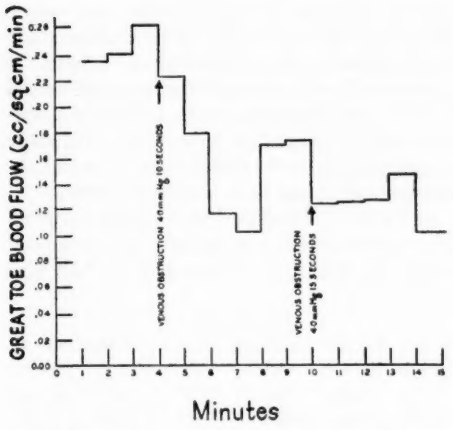

FIG. 1. REFLEX VASOCONSTRICTION PRODUCED BY VENOUS OCCLUSION

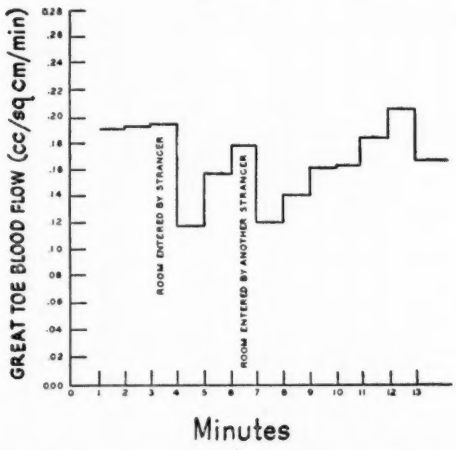

FIG. 2. THE EFFECT OF PSYCHIC STIMULI ON DIGITAL BLOOD FLOW (TOE)

pared with blood flow measured during spinal anesthesia in 12 patients. Spinal anesthesia was administered to these patients for surgical operations such as hernioplasty or hemorrhoidectomy. Anesthesia was considered complete when the patient became insensitive to pin prick and was unable to move his lower extremities. One additional patient suffering from causalgia, was studied pre-and two days post-sympathectomy.

RESULTS

The changes in blood flow after arterial and venous obstruction, in typical experiments, are presented graphically in Figures 3A and B. The results in many experiments on upper as well as lower extremities (Tables I and II) confirmed these findings. It will be seen that venous obstruction for several minutes decreased blood flow only slightly, and sometimes not at all; whereas, arterial obstruction reduced blood flow in the fingertip or toe sharply, although not quite to the zero level. The heat of metabolism was calculated

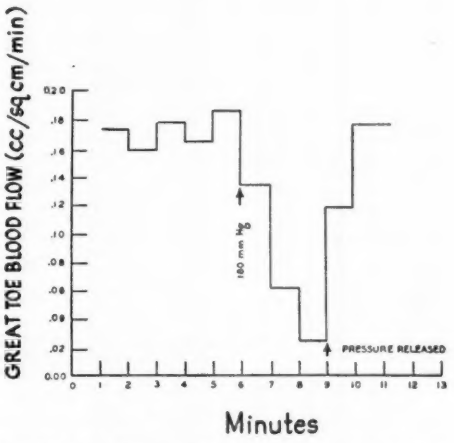

FIG. 3A. THE EFFECT OF ARTERIAL OCCLUSION ON DIGITAL BLOOD FLOW (TOE)

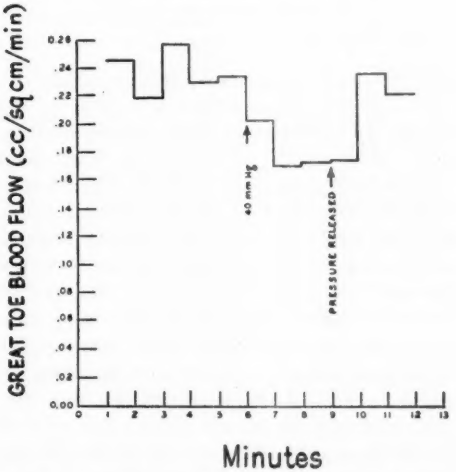

FIG. 3B. THE EFFECT OF VENOUS OCCLUSION ON DIGITAL BLOOD FLOW (TOE)

TABLE I

The effect of arterial obstruction on digital blood flow

Digit	Time of obstruction	Flow before obstruction	Lowest flow during obstruction	Flow after obstruction	Maximum per cent change in flow
	min.	*cc./sq.cm./min.*	*cc./sq.cm./min.*	*cc./sq.cm./min.*	
Great Toe	9	0.15	0.01	0.16	−93
	6	0.18	0.02	0.15	−89
	6	0.16	0.02	0.14	−88
	3	0.17	0.03	0.16	−82
	3	0.18	0.07	0.21	−61
	3	0.17	0.02	0.17	−88
	3	0.26	0.09	0.25	−65
	3	0.16	0.06	0.20	−63
	3	0.18	0.04	0.20	−78
	3	0.15	0.04	0.15	−73
	3	0.17	0.07	0.12	−59
	2	0.18	0.07	0.23	−61
	2	0.19	0.06	0.17	−68
	½	0.26	0.21	0.23	−19
	½	0.23	0.18	0.23	−22
	½	0.27	0.17	0.25	−37
	½	0.20	0.14	0.20	−30
	½	0.27	0.20	0.26	−26
	½	0.17	0.12	0.17	−29
	½	0.24	0.17	0.27	−29
	½	0.14	0.11	0.15	−21
Finger	8	0.34	0.07	0.28	−79
	3	0.32	0.03	0.32	−91
	3	0.24	0.04	0.25	−83
	3	0.32	0.03	0.28	−91
	3	0.12	0.03	0.11	−75
	3	0.26	0.04	0.24	−85
	3	0.39	0.05	0.38	−87
	½	0.31	0.23	0.34	−26
	½	0.21	0.16	0.23	−29
	½	0.29	0.23	0.29	−21
	½	0.27	0.21	0.30	−22
	½	0.24	0.20	0.24	−17
	½	0.32	0.22	0.33	−31
	½	0.30	0.22	0.33	−27

to be too small to be measured calorimetrically (3) and hence was not considered responsible for the residual heat elimination during arterial obstruction. With digital vasodilatation, some blood reached the fingertip or toe, probably via deep bone channels, even when the arteries were obstructed at several levels. To obliterate blood flow through the fingertip or toe completely, it was necessary to supplement arterial obstruction at the level of the ankle and toe, or at the level of upper arm, wrist and finger, by reflex vasoconstriction for several minutes (3). Several experiments, in which venous and arterial obstruction were employed for only 30 seconds or less, also revealed less decrease in flow during venous than during arterial obstruction, although the differences were less striking. The "lag" factor of the digital calorimeter, however, is approximately one minute and each of the series of flow determinations made in any patient covers a period of one minute. It would therefore be expected that such differences as occur with obstruction over a fraction of a minute would be "damped" to some extent. The lower venous pressure in the initial phase of venous obstruction might also be a "damping" factor.

In Table III, blood flow in the great toe measured calorimetrically after indirect heating supplemented by TEAC is compared with blood flow

TABLE II

The effect of venous obstruction on digital blood flow

Digit	Time of obstruction	Flow before obstruction	Lowest flow during obstruction	Flow after obstruction	Maximum per cent change in flow
	min.	*cc./sq.cm./min.*	*cc./sq.cm./min.*	*cc./sq.cm./min.*	
Great Toe	9	0.23	0.14	0.24	−39
	4	0.21	0.15	0.30	−29
	4	0.08	0.05	0.08	−38
	4	0.17	0.14	0.18	−18
	3	0.15	0.12	0.14	−20
	3	0.05	0.04	0.09	−20
	3	0.17	0.11	0.15	−35
	3	0.23	0.17	0.23	−26
	3	0.15	0.16	0.16	+ 7
	3	0.25	0.18	0.28	−28
	2	0.23	0.23	0.26	0
	2	0.13	0.08	0.10	−38
	2	0.21	0.18	0.24	−14
	2	0.16	0.13	0.16	−19
	2	0.16	0.13	0.15	−19
	½	0.06	0.06	0.06	0
	½	0.15	0.12	0.16	−20
	½	0.12	0.13	0.13	+ 8
	½	0.19	0.18	0.18	− 5
	½	0.28	0.12	0.30	−57
	½	0.19	0.15	0.19	−21
	½	0.14	0.14	0.13	0
	½	0.11	0.11	0.11	0
	½	0.17	0.17	0.17	0
	½	0.16	0.16	0.18	0
Finger	3	0.28	0.26	0.32	−32
	3	0.41	0.22	0.51	−46
	3	0.16	0.06	0.12	−63
	3	0.20	0.18	0.22	−10
	3	0.38	0.24	0.39	−37
	3	0.15	0.08	0.14	−47
	3	0.23	0.18	0.19	−22
	½	0.30	0.26	0.28	−13
	½	0.24	0.21	0.24	−13
	½	0.35	0.31	0.38	−11
	½	0.42	0.36	0.41	−14
	½	0.16	0.15	0.16	−06
	½	0.26	0.23	0.26	−12
	½	0.28	0.24	0.28	−14
	½	0.39	0.36	0.40	−08
	½	0.25	0.21	0.23	−16
	½	0.25	0.22	0.25	−12
	½	0.29	0.25	0.29	−14
	¼	0.28	0.22	0.27	−21

TABLE III

Patient	Age and sex	Indirect heating and TEAC		Spinal anesthesia or sympathectomy		
		Brachial blood pressure	Blood flow	Dosage pontocaine	Brachial blood pressure	Blood flow
		mm. Hg	*cc./sq.cm./min.*		*mm. Hg*	*cc./sq.cm./min.*
1	28 M	130/80	0.15	13 mg.	143/85	0.15
2	60 M	128/100	0.19	20 mg.	100/80	0.12
3	15 M	108/64	0.25	8 mg.	118/60	0.08
4	59 F	204/84	0.14	12 mg.	160/80	0.14
5	66 M	132/60	0.15	15 mg.	95/75	0.10
6	48 F	108/64	0.18	16 mg.	100/70	0.14
7	55 F	96/70	0.16	15 mg.	95/75	0.19
8	42 M	92/76	0.21	16 mg.	110/70	0.10
9	24 M	88/70	0.14	15 mg.	90/60	0.15
10	44 F	112/72	0.23	15 mg.	85/70	0.17
11	29 M	82/52	0.15	16 mg.	80/50	0.07
12	29 M	116/80	0.15	12 mg.	108/80	0.20
13	22 M	130/90	0.21	2 days after sympathectomy	110/72	0.19

during spinal anesthesia in 12 cases and after sympathectomy in one case. With the calorimetric technique blood flow during spinal anesthesia or after sympathectomy was no greater, and in many cases considerably less, than after indirect heating reinforced by TEAC. A decreased systemic blood pressure or circulating vasoconstrictive substances, such as adrenalin, released by the apprehension prior to an operation, might account for the depression of blood flow during spinal anesthesia in those cases in which it occurred. Page, Prince, and Reinhard (11) have shown that circulating vasoconstrictive substances are released from the adrenal medullae and the liver of animals by the intravenous administration of large doses of TEAC. Since the normal range of variation in blood flow is shifted only slightly by supplementing indirect heating by the administration of TEAC intravenously (4) it is unlikely that significant humoral vasoconstriction was induced in our patients by TEAC, in the dosage used.

DISCUSSION

It is apparent from these experiments that prolonged venous obstruction is incomplete because of considerable reflux of blood probably via deep veins. This reflux may occasionally be great enough to accommodate the total influx from the arteries. Even with 30 seconds of venous obstruction, all the blood is not trapped in the finger or toe. We have no evidence bearing on the first five or 10 seconds of venous obstruction where digital plethysmographic slopes are usually drawn. The calorimetric lag is too great to make successful comparisons of flow at such short intervals. It must be pointed out, however, that reflux of blood may occur even during this initial phase of obstruction, and may produce a variable error in the measurement of blood flow. It has also been demonstrated (12) that at high rates of flow increased pulse volume makes it more difficult to draw accurate digital plethysmographic slopes. In contrast, it is at such high rates of flow that the digital calorimeter becomes most reliable (3). In addition to these factors, it is clear that reflexes induced psychically, or by the very act of venous obstruction, may "break through" the release of sympathetic tone by heating alone and less commonly by heating together with intravenously administered TEAC to distort flow measurements dependent on such obstruction. Since it is less likely that such reflexes can "break through" spinal anesthesia, this factor would also confuse comparison between procedures for releasing sympathetic nerve tone.

In the studies with the foot plethysmograph (7) caudal anesthesia or sympathectomy was compared with heat or TEAC rather than a combination of the two. The combination may be more effective than either of these agents alone in releasing sympathetic nerve tone in the lower extremities. What is more, there may be differences between the reactions of deep blood vessels and those of the skin (7) making comparison between the results of foot plethysmography and digital calorimetry difficult. The fact, however, that spinal anesthesia is no more effective than indirect heating supplemented by TEAC in releasing sympathetic nerve tone, if the calorimetric technique is used, indicates that the latter procedure releases such tone effectively at least in the skin of the toe.

SUMMARY AND CONCLUSIONS

Spinal anesthesia produces no greater release of sympathetic nerve tone in the great toe than indirect heating supplemented by TEAC when blood flow in the skin is measured calorimetrically. Factors responsible for discrepancies between plethysmographic and calorimetric data were investigated and are discussed.

ACKNOWLEDGMENT

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XL. QUANTITATIVE SEPARATION AND DETERMINATION OF THE PROTEIN COMPONENTS IN SMALL AMOUNTS OF NORMAL HUMAN PLASMA [1]

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The need for improved methods for the determination and characterization of the protein components of human blood plasma requires no emphasis. The commonly employed methods for the separation of plasma proteins on the basis of solubility give little detailed information and generally yield poor separations (1). Likewise, the method of electrophoretic analysis, although of unquestioned value, will fail to differentiate between components of widely differing chemical properties and physiological functions if they happen to have the same mobility in an electric field.

The present communication describes the adaptation of Method 10 of plasma fractionation developed by Cohn and his associates (2) for the quantitative estimation of certain of the protein and lipoprotein components in small quantities of normal human plasma. The fractionation is carried out at low temperatures using low concentrations of ethanol and of various salts, resulting in the concentration of each protein component in an undenatured state almost exclusively in one fraction or another.

In the present system of analysis, samples of 5 ml. of plasma have been separated by filtration into four primary fractions (Figure 1). The concentrations of the serum albumins, γ-globulins, α-lipoproteins, and β-lipoproteins were measured, together amounting to about 75% of the total proteins of normal human plasma. Data have also been obtained on the distribution of protein-bound carbohydrate (hexose) among the fractions, but the information is not yet available which would make it possible to calculate the quantities of specific glycoproteins and mucoproteins.

Although the present communication deals with the determination of only four major components of normal plasma, the system of separation has been designed to allow the incorporation of procedures for the isolation and measurement of other components as soon as methods have been worked out. In addition to quantitative analysis of the major protein components in the plasma the proposed method will facilitate the quantitative estimation of trace components, because each fraction contains certain components in higher concentration and in simpler mixtures than whole plasma. Because the proteins are present in various fractions in an unaltered state their biological activities remain unimpaired and can be measured. The method can, furthermore, be used on abnormal plasmas for the quantitative determination of various proteins present in abnormal quantities in the plasma of patients with various diseases. Such quantitative analyses of abnormal plasmas are being carried out at present and will be published in a future communication. Recognition and characterization of pathological proteins, as

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they are present in several diseases, such as multiple myeloma and lupus erythematosus, will be facilitated by this method.

The method has been designed to be as simple and rapid as possible without sacrificing accuracy. All operations, including the separations of precipitates by filtration under vacuum, are carried out in the same cold bath. The separations of six samples of plasma into the four primary fractions can normally be completed in one day by a single worker. The buffer solutions employed were chosen so that for each step stock reagents are added in standard amounts without the necessity of performing tedious pH adjustments. Accurate separations have been made possible by the use of liberal quantities of buffer solutions to wash the precipitates on the filters. The use of the buffer solutions for washing has the added advantage that small errors in the pH adjustment at any one step are corrected before subsequent separations are undertaken, and in this way such errors are prevented from accumulating.

SYSTEM OF PLASMA ANALYSIS

Method 10 of plasma analysis employs a system in which five or more variables are critically controlled: pH, ethanol concentration, ionic strength, protein concentration, temperature and, in some instances, concentration of dipolar ions or of divalent metallic or organic ions.[5] The conditions at which these variables are fixed, and the components known to be concentrated in the different fractions are given in Figure 1.

In the *first step* of the procedure, many of the largest and least soluble of the plasma proteins are rendered completely insoluble, and are separated in Fraction I + II + III. The extremely low solubility of the proteins of Fraction I + II + III under the conditions of this separation is due in part to the formation of insoluble salt-like complexes between proteins of opposite net charge (2). The γ-globulins, most notably, owe their insolubility to combination with β-lipoproteins and other α-globulins and β-globulins, including caeruloplasmin. In a sense, the components naturally present in the plasma are used as reagents to effect their own quantitative separation. It will be shown below that wide variations in the concentrations of β-lipoproteins and γ-globulins do not interfere with the completeness of the separation of Fraction I + II + III from Fraction IV + V + VI.

The *second step* of the procedure involves the precipitation in Fraction IV + V of nearly all the proteins which remained in solution after the initial precipitation of Fraction I + II + III. In this way almost all the plasma proteins are reduced to the more stable solid state within a short time after the beginning of the fractionation. The precipitation is effected by the addition of a small amount of zinc acetate without changing the other variables. The proteins precipitated as insoluble zinc salts include the serum albumins, β_1-metal combining protein, α_2-glycoproteins, α_2-mucoproteins, α-lipoproteins, iodoproteins, choline esterase and alkaline phosphatase. The zinc salts are readily soluble in distilled water and in a variety of buffer solutions. Analytical determinations for serum albumins and α-lipoproteins were usually made on redissolved Fraction IV + V, although for certain purposes measurements could be made directly on the solution of Fraction IV + V + VI. However, for the determination of protein-bound carbohydrate (as hexose) it is necessary to separate Fraction IV + V and then to precipitate Fraction VI, leaving the free carbohydrate (blood sugar) in solution. Serum albumins are estimated as methemalbumin (5), and the α-lipoproteins from determinations of cholesterol and lipid phosphorus.

Fraction VI, which remains in solution after the precipitation of Fraction IV + V, contains traces of the proteins of Fraction IV + V, and in addition an α_1-acid glycoprotein, small amounts of α_2-globulins and β_1-globulins, as well as other small proteins and peptides, urea, and glucose. The protein and certain peptide constituents of this fraction are precipitated by the addition of barium acetate and an increase in the concentration of ethanol.

The *third step* of the procedure serves to extract from Fraction I + II + III almost all the γ-globulins in a nearly pure form, as judged by electrophoretic analysis of Fraction II. To accomplish this separation, it was found necessary to add the dipolar ion glycine to decompose the complexes in which the γ-globulins are held. The ethanol concentration of 15% and the low ionic strength used

[5] For more detailed information about the role of these variables in protein separations, see Cohn and his colleagues (2, 3) and the review by Edsall (4).

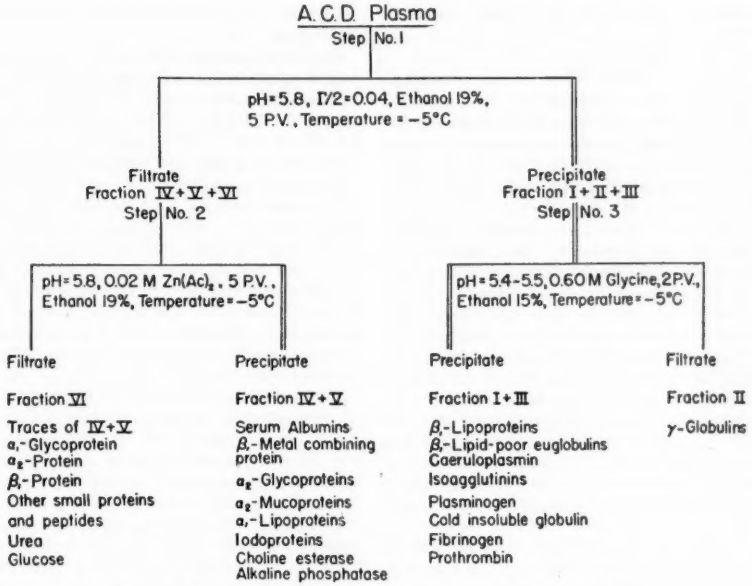


FIG. 1. PROTEIN COMPONENTS CONCENTRATED IN THE FOUR PRIMARY FRACTIONS

during the extraction prevents solution of more than traces of β-globulins.

The yield of protein in Fraction II is obtained by direct measurement with the biuret reaction on an aliquot of the solution. When concentration of the extract is required prior to electrophoretic, ultracentrifugal, or other analyses, the γ-globulins are quantitatively reprecipitated by the addition either of sodium tartrate or of a mixture of sodium sulfate and zinc acetate, accompanied by an increase in concentration of ethanol. The use of the divalent anions makes it unnecessary to increase the ethanol concentration to the point where glycine would precipitate along with the γ-globulins. The precipitates obtained in this way are readily soluble in distilled water or a wide variety of salt solutions.

Fraction I + III, which remains insoluble after the extraction of the γ-globulins, contains β-lipoproteins, caeruloplasmin, isoagglutinins, most of the components concerned with blood clotting, and a number of α-globulins and β-globulins whose characteristics are not yet well understood. The use of the technique of fractional extraction for the removal of the γ-globulins serves to keep the components of Fraction I + III insoluble from the time that they are precipitated in the initial step of the procedure until they are redissolved for analysis at the very end of the operation. In the solid state these components are quite stable and are protected from various changes, chemical and enzymatic, which occur rapidly in solution. The yield of β-lipoproteins in Fraction I + III is estimated from cholesterol and lipid phosphorus determinations on the redissolved fraction.

MATERIALS AND METHODS

Collection of Blood

Blood was obtained from male subjects by venipuncture with a syringe containing 1.5 ml. of acid citrate dextrose solution (A.C.D.) for each 8.5 ml. of blood to be drawn. The blood cells were removed by centrifugation to obtain the plasma, designated as "A.C.D. plasma," which was used for the separations and analyses.[6] The acid citrate dextrose solution contained per liter 26.7 g. trisodium citrate ($5\frac{1}{2}$ H_2O), 8.0 g. citric acid monohydrate, and 22 g. dextrose. At the same time, for the purpose of a reference determination of total protein, approximately 5 ml. of blood were drawn with a dry syr-

[6] The tendency of the clot to entrap certain proteins to a larger extent than others prompted our decision not to apply the separation method to serum (6).

inge, and placed in a tube containing a small amount of heparin powder.

Apparatus

The fractionation was carried out in a $-5°$C. bath containing 50% by volume of ethanol, provided with an effective stirrer, and deep enough to submerge the filtration apparatus to the required depth.[7] For the filtration a sintered glass filter cup with a maximum porosity of 14 microns and a capacity of 60 ml. was used.[8, 9] This filter cup was connected to a 100 ml. round-bottom collecting tube by a two-hole rubber stopper. The glass stem of the filter cup entered the collecting tube through one hole of the stopper. A suction system was connected through the other hole of the stopper. The filter cups and attached collecting tubes were held in the bath by a rigid amount of sufficient size to accommodate 12 such filtration units. The availability of 12 filtration units allowed the processing of six specimens of blood plasma in one day.

A water aspirator was used to obtain vacuum. A regulating column of mercury was employed to control the vacuum,[10] and a large carboy was placed in the suction line to minimize pressure variations due to the aspirator. A steady pressure of 38 cm. of mercury was found most effective.

Adequate cleaning of the glass filter cups as soon as possible after use is important. They were first washed with soap and water. Thereafter, saturated borax solution, dilute hydrochloric acid, distilled water, and 95% alcohol were drawn successively through the filters under negative pressure provided by a water aspirator. (If the filters were still plugged, several lots of a 2% aqueous solution of duponol were drawn through the filters prior to the use of the saturated borax solution.) It was found inadvisable to use any one glass filter cup for more than ten runs.

Reagents

All reagents were freshly prepared from stock solutions at room temperature and precooled to $-5°$ (or the freezing point of the mixture) before use. The stock solutions were made with C.P. grade reagents without further purification.

Reagent A contained 250 ml. 95% ethanol, and 2.4 ml. of sodium acetate buffer, and water to make 1 L. The stock sodium acetate buffer consisted of 200 ml. of 4 M sodium acetate and 400 ml. of 10 M acetic acid per L. This buffer diluted with distilled water 80 times had a pH of 4.00 ± 0.02 in a glass electrode at 25° ([3] footnote 68).

Reagent A' contained 200 ml. of 95% ethanol, 40 ml. of 1 M sodium acetate, 3.5 ml. of 1 M acetic acid, and water to make 1 L. The pH was 5.80 ± 0.05.

Zinc Reagent contained 54.8 g. of zinc acetate dihydrate which was first dissolved in about 500 ml. of water; to this solution 200 ml. of 95% ethanol were added, and then water to make 1 L. The solution should be kept at refrigerator temperature since at room temperature undesirable deposition of insoluble material occurs. The pH of this solution was 6.5 ± 0.1.

Barium Reagent contained 10.22 g. barium acetate, 640 ml. 95% ethanol, and water to make 1 L.

Reagent B contained 150 ml. 95% ethanol, 45 g. glycine, 3.8 ml. 1 M sodium acetate, 2.6 ml. 1 M acetic acid, and water to make 1 L. The pH was 5.20 ± 0.02.

Reagent B' contained 150 ml. of 95% ethanol, 45 g. glycine, 5 ml. of 1 M sodium acetate, 1.8 ml. 1 M acetic acid, and water to make 1 L. The pH was $5.40 - 5.45$.

Citrate Reagent contained 9.0 g. sodium chloride, 7.1 g. trisodium citrate ($5\frac{1}{2}$ H_2O), and water to make 1 L.

Tartrate Reagent contained 13.8 g. disodium tartrate, 475 ml. 95% ethanol, and water to make 1 L.

Sulfate Reagent contained 8.5 g. anhydrous sodium sulfate, 4.4 g. zinc acetate dihydrate, 362 ml. of 95% ethanol, and water to make 1 L.

Separation Procedures

Step No. 1. The initial precipitation of Fraction I + II + III, leaving Fraction IV + V + VI in solution, was performed as follows. Two ml. of precooled Reagent A were placed in the sintered glass filter cup which was immersed in the cold bath within 1 cm. of the lip. Five ml. of A.C.D. plasma precooled to 0–2° were added dropwise with stirring, followed by 18 ml. of Reagent A again added dropwise with good stirring.[11] After the addition was completed, the mixture was stirred for five minutes, and allowed to stand for 10 to 15 minutes before the vacuum was applied. The filtration required about 40 minutes.

The precipitate was then washed[12] by resuspending it

[7] A 50 gallon capacity cold bath supplied by the American Instrument Company, Silver Spring, Md. was used. The internal dimensions are $21.75 \times 37.75 \times 15$ inches, and the temperature was adjustable to within 0.5°C. over a temperature range between room temperature and $-29°$.

[8] These filter cups are made by the Corning Glass Works, and were obtained from Macalaster-Bicknell Co., Cambridge, Mass. Filter cups with a maximum porosity of 14 microns are marked M; those with a maximum porosity of 5 microns are marked F.

[9] Very rarely, it was found that the precipitate formed by the initial precipitation would pass through the filter. In such instances a sintered glass filter cup with a maximum porosity of 5 microns was used to obtain a clear filtrate.

[10] In recent work, satisfactory vacuum regulation has been obtained with a Cartesian Manostat No. 6, supplied by Emil Greiner Co., 22 N. Moore St., New York, N. Y.

[11] If the plasma was placed in the filter cup before any reagent was added, it was found that some of the plasma penetrated the pores of the filter before precipitation occurred. This resulted in considerable slowing of the filtration due to freezing of some of the plasma in the filter pores, and cloudiness of the filtrate due to the escape of some precipitable proteins through the filter. On the other hand, if larger volumes of reagent were added first, the plasma would be exposed initially to excessively acid conditions.

[12] Without washing, appreciable quantities of the soluble proteins belonging to Fraction IV + V + VI remained

in 25 ml. of Reagent A'. The precipitate was at first stirred to a thick uniform paste with a small amount of the reagent before the rest of the reagent was added with constant stirring. The collecting tube containing Fraction IV + V + VI was left in place to receive the washings. The vacuum was applied without delay. The filtration required about 30 minutes.

Step No. 2. As soon as the filtration of the washing had been completed, the filtration apparatus was momentarily removed from the cold bath, the receiving flask transferred to a wire basket suspended in the cold bath, and a fresh receiving flask attached in its place. The filtration apparatus was immediately reimmersed. To the solution of Fraction IV + V + VI in the first receiving flask were added 4 ml. of the Zinc Reagent, causing copious precipitation. The resulting suspension was allowed to stand for 15 to 30 minutes in the cold bath (overnight was found permissible), and was then poured into a new filter cup which differed from the first only in that the maximum porosity was 5 microns instead of 14 microns. The last traces of the suspension in the first receiving flask were washed into the filter cup with the aid of a few ml. of 0.02 M zinc acetate dissolved in 19% ethanol. Filtration was carried out as before. The precipitate remaining on the filter constituted Fraction IV + V, and was redissolved either in 0.15 M sodium chloride or 0.02 M sodium citrate to a volume of 5 ml. for the purpose of analysis.

The filtrate, Fraction VI, contained so little protein that frequently it was not subjected to further isolation procedures. When desired, the proteins of Fraction VI were precipitated by the addition of an equal volume of Barium Reagent, and separated as before by filtration through a glass filter with a maximum porosity of 14 microns. The precipitate was washed with two 10 ml. portions of the following reagent: 5.47 g. barium acetate monohydrate, 4.39 g. zinc acetate dihydrate, 421 ml. 95% ethanol, and water to make 1 L. The precipitate was then dissolved in a mixture of 0.5 ml. Citrate Reagent and 4.5 ml. distilled water, and the resulting solution drawn through the filter. The filter was washed through with 2 ml. of 0.1 M sodium sulfate solution and the washings added directly to the dissolved Fraction VI. The Fraction VI solution was made up to 10 ml. with distilled water, and placed in a refrigerator for 30 minutes to allow the precipitate of barium sulfate to form. The precipitate was removed by a short centrifugation in an ordinary laboratory centrifuge before aliquots of the clear solution were taken for biuret and hexose analyses. If only the biuret determination was required, it was sufficient to dissolve the precipitate of Fraction VI in the diluted Citrate Reagent.

Step No. 3. The third step of the separation procedure involved the extraction of Fraction II from Fraction I + II + III, leaving Fraction I + III as residue. This separation was carried out concurrently with Step No. 2. Fraction I + II + III in the original filter cup was resuspended in 25 ml. of Reagent B. As in the previous washing with Reagent A', only about 1 ml. of Reagent B was added at first, and a thick, uniform paste was prepared. Thereafter, the rest of the reagent was added slowly with constant stirring. The suspension was allowed to stand for one hour with occasional stirring before the vacuum was applied. The filtration required approximately two hours.

The residue was then washed by resuspending it in 10 ml. of Reagent B'. Filtration was started without delay. The washings were filtered into the same collecting tube which contained Fraction II. This filtration required only about 10 minutes.

The residue on the filter cup, representing Fraction I + III, was removed as completely as possible with a small stainless steel spatula. Three small portions of Citrate Reagent were drawn through the filter in order to remove any protein remaining in the pores. These effluents were used to dissolve the paste previously removed with the spatula, and the whole was made up to 5 ml. with Citrate Reagent and taken for analysis. Allowing the suspension to warm slightly by exposing it to room temperature facilitated solution.[13]

Reprecipitation of the protein components of Fraction II was effected by adding 35 ml. of Sulfate Reagent to the Fraction II filtrate and washings. If for subsequent assays the presence of zinc ions was undesirable, and their removal or neutralization inconvenient, 35 ml. of Tartrate Reagent was substituted for the Sulfate Reagent.[14] In either case, the precipitate was allowed to form for one hour before removal by filtration in the cold bath through a glass filter cup with a maximum porosity of 14 microns. The filtration required about 15 minutes. The precipitated Fraction II was dissolved and made up to 5 ml. with distilled water or 0.15 M sodium chloride.

Analytical Methods

Nitrogen determinations were carried out by the micro-Kjeldahl method. Non-protein nitrogen (NPN) was determined after precipitation of the proteins with 10% trichloracetic acid.

Biuret determinations were carried out by a modification of the method of Mehl (7) in which each series of readings was standardized against a standard preparation of normal human serum albumin ([6] footnote 14).[15] Determinations were made by diluting each aliquot to 10 ml. with 0.15 M sodium chloride before addition of 1

occluded in Fraction I + II + III and appeared subsequently in Fraction II, resulting in contamination of the γ-globulins in Fraction II to the extent of about 20% with albumins. Carrying out the initial precipitation at higher dilutions did not circumvent this difficulty as satisfactorily as did the introduction of the washing step.

[13] A small amount of material often failed to dissolve.

[14] When the Tartrate Reagent was employed, precipitation of the proteins was only about 95% complete, but could be made quantitative by carrying out the filtration at $-8°$.

[15] Crystalline bovine serum albumin, obtainable from Armour and Co., Chicago, Ill., may be used in place of the human serum albumin without the need of applying any corrections due to the species difference.

ml. of the biuret reagent. The reagent was prepared as follows. To a solution of 20.5 g. cupric chloride dihydrate in 300 ml. of water, 150 ml. of ethylene glycol was added, followed by 300 g. of sodium hydroxide in aqueous solution, and the whole was diluted to 1 L. with water and transferred to a large Erlenmeyer flask covered with a watch-glass, and heated for four hours on a steam bath. The solution was filtered after cooling. Color development was allowed to proceed for 20 to 30 minutes before measurement in a Leitz photoelectric colorimeter set at 550 mμ. (Klett and Coleman instruments have also been employed.)

Lipid analyses were carried out on ethanol-ether extracts prepared as follows. The protein solutions were added to about 20 volumes of ethanol-ether mixture (3:1 by volume), heated to boiling, cooled, and made up to 25 volumes. Aliquots were taken after filtration for the determination of total cholesterol and lipid phosphorus. *Total cholesterol* was determined by the method of Bloor, Pelkan and Allen (8). The photoelectric colorimeter was set at 640 mμ. *Lipid phosphorus* was determined by the method of Fiske and SubbaRow (9) following the ashing procedure of Gortner (10). Color intensity was measured at 640 mμ. The factor 25.0 was used to convert lipid phosphorus to phospholipid.

Serum albumin determinations were carried out by the hemin-binding method of Rosenfeld and Surgenor (11).

Hexose determinations were made according to a modification of the method of Sørensen and Haugaard (12) introduced by Friedmann (13). The reaction mixture contained 1 ml. of protein solution, 2.5 ml. of 1.6% orcinol in 50% sulfuric acid (by volume) and 15 ml. of 60% sulfuric acid. Color was developed by heating for 20 minutes at 80°, cooling in ice in the dark for 20 minutes, then standing at room temperature in the dark for 30 minutes. The color was measured at 445 mμ. An equimolar mixture of galactose and mannose was used to prepare the standards included in each series of determinations.

Fibrinogen determinations (clottable protein) were made according to the method of Morrison (6).

Determinations of pH were carried out in the glass electrode at 25°, using 1 ml. of reagent solution or of protein suspension mixed with 4 ml. of 0.02 M sodium chloride.

Electrophoretic analysis of plasma and of the various fractions was used for supplementary characterization of the various fractions. The measurements were carried out in a sodium diethyl barbiturate-sodium citrate buffer at pH 8.6 and an ionic strength of 0.1 by the standard procedure employed in this laboratory (14). The buffer contained per L. 11.05 g. of barbital, 2.45 g. of sodium citrate ($2H_2O$) and 50 ml. of 1N NaOH.

Determinations of dry weight, as a method of total protein determination, were carried out as follows:[16]

A length of cellophane tubing 0.5 inch in diameter was weighed, soaked in water, and a knot was tied in one end. A known volume of the solution to be analyzed containing 100 to 300 mg. protein was added from a carefully wiped pipette introduced well down inside the bag. A small volume of distilled water was used to wash down any protein adhering near the opening of the bag. The bag was tied with sufficient slack to avoid squeezing the protein solution above the knot and to allow room for expansion on freezing. At the same time an equal length of tubing was tared and filled with an equal volume of distilled water. The two bags were dialyzed for three to four days against several daily changes of distilled water. The bags were then frozen rapidly by immersion in 95% ethanol cooled by a bath of dry ice in ethanol. The frozen bags were immediately transferred to a vacuum oven connected to a dry ice-ethanol trap and vacuum pump. The temperature of the oven was immediately raised to 75°. Constant weight was reached within 12 hours.

The initial weight of the tubing containing the unknown sample was corrected on the assumption that it underwent the same proportionate decrease in weight on drying as the control.

Neutralization or removal of zinc or barium ions added in performing the separations was found necessary for some assays. This was accomplished by one of the following methods. For many purposes it was found sufficient to neutralize the effect of the metals by the addition of sodium citrate or the more effective ethylenediaminetetraacetate[17] without actually removing the metals from the solution. When complete removal of the added cations was desirable, Fraction IV + V, or VI, was dissolved in water and passed through a cationic exchange resin column at 0°. A column of Dowex-50 exchange resin[18] 10 cm. in height and 2 cm. in diameter resulted in removal of usually more than 99% of the added divalent metals when the protein solution was allowed to pass through at the rate of 0.25 ml./cm.²/min. Since it is necessary that the resin column be covered with liquid at all times, liberal quantities of distilled water must be passed through the column to displace the protein solution. When removal of the last traces of metals was not required, it was sufficient to stir the protein solution directly with the resin and to wash by decantation.

Analysis of Plasma and Separated Fractions

The analytical procedures were applied to the various fractions as follows:

[16] We are indebted to Dr. M. A. Lauffer, University of Pittsburgh, for the general description of this method.

[17] The trisodium salt of ethylenediaminetetraacetic acid was supplied as Sequestrene NA3 by the Alrose Chemical Co., Providence, R. I.

[18] The cationic exchange resin, Dowex-50 (plant grade, 30–50 mesh) may be obtained on the sodium cycle from National Aluminate Corp., Chicago 38, Ill., under the name Nalcite HCR. The only preparation necessary consisted in washing the resin by swirling in a flask with successive volumes of hot distilled water and allowing it to settle. This process was continued until the water above the settled resin was clear. The resin may be reactivated by treating for 15 minutes with 0.15 M sodium chloride, followed by thorough washing with distilled water.

Plasma

a) Total protein was calculated by applying the factor 6.73 to the value for protein nitrogen (15).[19] To correct the values obtained on the A.C.D. plasma (*i.e.*, plasma with acid citrate dextrose as anticoagulant) to absolute values, the nitrogen determination was repeated on plasma from heparinized blood drawn at the same time.[20] The factor (N in heparinized plasma/N in A.C.D. plasma) has been applied to all data for A.C.D. plasma and the various fractions.[21]

b) Total cholesterol and lipid phosphorus were determined on an aliquot of 2 ml.

c) Fibrinogen was determined on an aliquot of 1 ml.

d) Serum albumin was occasionally determined on an aliquot of 1 ml. made up to 125 ml. with sodium phosphate buffer of pH 7.3 and ionic strength 0.3.

Fraction IV + V

a) Total protein was determined by the biuret reaction on an aliquot of 0.5 ml.

b) Total cholesterol and lipid phosphorus were determined on an aliquot of 2 ml. The value obtained for total cholesterol was converted into the value for α-lipoproteins by multiplying by 6.25.[22]

c) Serum albumin was determined on an aliquot of 1 ml. made up to 100 ml. with sodium phosphate buffer of pH 7.3 and ionic strength 0.3.

d) Hexose was determined on an aliquot of 1 ml.

Fraction VI

a) Total protein was determined by the biuret reaction on an aliquot of 5 ml.

b) Hexose was determined on an aliquot of 2 ml.

Fraction IV + V + VI

Occasionally the solution of Fraction IV + V + VI was analyzed as a whole for total protein, total cholesterol, lipid phosphorus and serum albumin before Step No. 2 was carried out. The presence of the free carbohydrate (glucose) of plasma in Fraction IV + V + VI made impossible hexose determinations before separation of the glucose by washing Fraction VI.

Fraction II

a) Total protein was determined by the biuret reaction on an aliquot of 5 ml. of the extract of Fraction II before precipitation. Five ml. of Reagent B′ were included in the standard and blank biuret reagent mixtures to allow for the small enhancement of optical density due to the formation of copper-glycine complexes. Alternatively, 2 ml. of the redissolved Fraction II solution were used without the necessity of making allowance for the interference of glycine.

b) Total cholesterol and phospholipid were determined on an aliquot of 3 ml. of the redissolved Fraction II.

Fraction I + III

a) Total protein was determined by the biuret reaction on an aliquot of 1 ml.

b) Total cholesterol and lipid phosphorus were determined on an aliquot of 2 ml. The value obtained for total cholesterol was converted into the value for β-lipoproteins by multiplying by 2.86.[23]

For the calculation of the total protein in Fraction I + III, the value for the biuret determination was corrected by the addition of the quantity (g. per 100 ml. of cholesterol) × 2.14, based on the presence of 75% total lipids in the β-lipoproteins (17). On the average such a correction gave a ratio of total protein to biuret value of 1.27. This calculation may be expected to fail when applied to the fraction separated from lipemic plasma or plasma containing highly atypical lipoproteins. The necessity for including the lipid correction was shown by comparison of the value for total protein obtained by dry weight with the biuret value. For a typical Fraction I + III, the ratio of dry weight to biuret value was 1.19, in contrast to ratios of 1.02 and 0.97 for Fractions IV + V and II, respectively. Because of the small deviations, it

[19] In the usage of this Laboratory, "total protein" is synonymous with "total non-dialyzable solids." This practice allows a more direct interpretation of electrophoretic and ultracentrifugal data than the use of some purely arbitrary value such as N × 6.25, since the contribution of the lipids to the refractive index of plasma or of the fractions is only a little less than that of a corresponding weight of protein (14). The theoretical justification of such a practice is that in normal fasting plasma all, or almost all, of the lipids appear to be present in the form of well-defined lipoproteins, and tally should be kept of the whole of such components rather than only of their peptide part (16, 17). The same argument applies to protein-bound sugar. The factor 6.73 is most accurately employed with pooled normal plasma but obviously will not, in general, be correct for highly abnormal plasmas. For this latter purpose, individual determinations of dry weight are recommended.

[20] The use of heparin as anticoagulant for the plasma to be fractionated was avoided on the advice of Dr. Alfred Chanutin who had observed that in such a case Fraction I + II + III tended to clot readily. The possibility that heparin would affect the solubility behavior of various plasma proteins was also a deterrent to its use.

[21] The value of this factor varied between 1.10 and 1.30, with an average of about 1.20. Comparison with the value of 1.32 which would be predicted (for a plasmacrit of 0.55) on the basis of simple dilution with the acid citrate dextrose solution shows a varying effect of the A.C.D. solution on the red cell volume.

[22] For this calculation the percentage of cholesterol in the α-lipoproteins was taken as 16 ([3] Table VIII, Fraction IV–1, 1).

[23] For this calculation the percentage of cholesterol in the β-lipoproteins was taken as 35, since it was found that the cholesterol determination employed in this procedure yielded 35% cholesterol instead of the average of 30.9% obtained with a more accurate procedure (16).

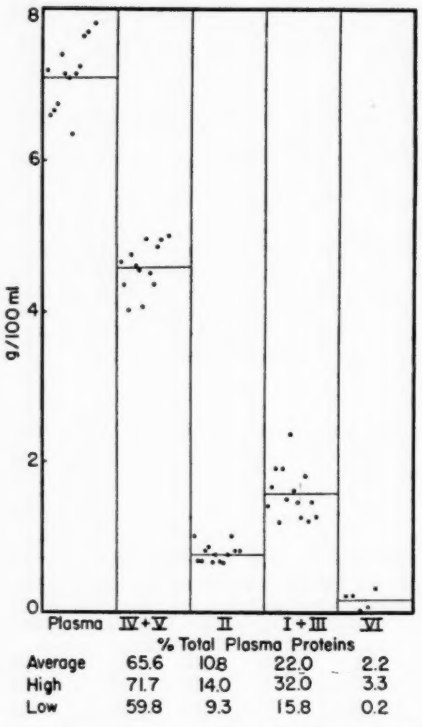


FIG. 2. CONCENTRATION OF TOTAL PROTEIN IN PLASMA AND IN THE FOUR PRIMARY FRACTIONS

has been considered unnecessary for routine purposes to apply corrections to the total protein values obtained by the biuret determinations on Fraction IV + V and II.

RESULTS

Protein Distribution

Representative data for the concentration of total protein in plasma and in each fraction are given in Figure 2. The average for each group of data is indicated by a horizontal line. Average and extreme high and low values for the per cent of the total plasma proteins in each fraction are shown at the bottom of the figure.

Cholesterol and Phospholipid Distribution

The distributions of cholesterol and phospholipid among the fractions are shown in Figure 3. The mole ratio of cholesterol to lipid phosphorus among the fractions is presented in Table I. The quantities of cholesterol and phospholipid separated in each fraction were slightly more variable than the total protein (Figure 2); yet the constancy of the distribution was still striking. Perhaps most interesting was the finding of the fairly constant and yet widely differing mole ratios of cholesterol to lipid phosphorus in Fractions IV + V and I + III, associated with α-lipoproteins and β-lipoproteins respectively (Table I).[24] Fraction IV + V, containing the α-lipoproteins, had significantly larger amounts of phospholipids than of cholesterol; whereas in Fraction I + III, containing the β-lipoproteins, the amount of cholesterol was greater than that of phospholipids. Some of the other contrasting properties of these two groups of lipoproteins have been described elsewhere (17). The high ratio of cholesterol to phospholipid found in Fraction II is characteristic of neither of the main groups of α-lipoproteins or β-lipoproteins, and invites further study to establish the form in which these lipids are present in Fraction II.

The proportion of the total cholesterol in the esterified form was determined on the fractions of six samples of plasma by the method of Schoenheimer and Sperry (18). The average values for the per cent esterified cholesterol were 73.4%, 70.1% and 76.7% for plasma, Fraction I + III, and Fraction IV + V, respectively. These results confirm the essential constancy of the re-

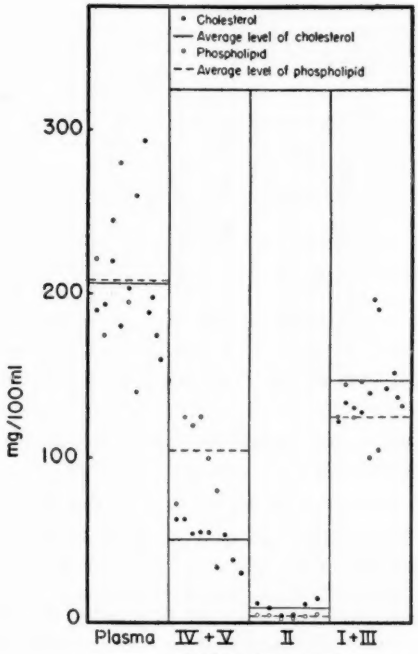
[24] The mole ratio in Fraction I + III, 2.31, is in agreement with the value of 2.11 calculated from the data of Oncley, Gurd and Melin (16) for the preparations of purified β-lipoproteins separated from large pools of normal plasma. The present values would have been slightly lower if the more tedious analytical procedure (involving hydrolysis of cholesterol esters) used by Oncley and his associates had been employed.

TABLE I

Mole ratio of total cholesterol to lipid phosphorus in plasma fractions

Mole ratio cholesterol/lipid P

	Fraction			Plasma
	I + III	II	IV + V	
	2.21	5.75	1.01	1.52
	2.33	4.10	0.72	1.63
	2.50	3.88	0.80	1.70
	2.31	3.90	0.72	1.72
	2.18	5.88	0.78	1.88
Average	2.31	4.70	0.81	1.69



	% Total Cholesterol		
Average	26.6	4.0	71.4
High	33.3	6.9	83.1
Low	18.0	1.5	54.8
	% Total Phospholipid		
Average	46.7	1.5	52.8
High	57.0	2.3	56.6
Low	33.0	0.8	51.0

FIG. 3. DISTRIBUTION OF CHOLESTEROL AND PHOSPHOLIPID AMONG THE FRACTIONS

lationship between esterified and unesterified cholesterol in the major lipoprotein fractions, first shown in fractions prepared by a forerunner of the present method (3).[25]

Fraction VI was found to contain at most immeasurably small traces of cholesterol and phospholipid.

Carbohydrate Distribution

The distribution of protein-bound carbohydrate (hexose) among the protein fractions is presented in Figure 4. The lack of information about the

[25] Personal communications from Drs. W. M. Sperry, M. Melin, and A. Chanutin. For more recent studies, see Pearsall and Chanutin (19).

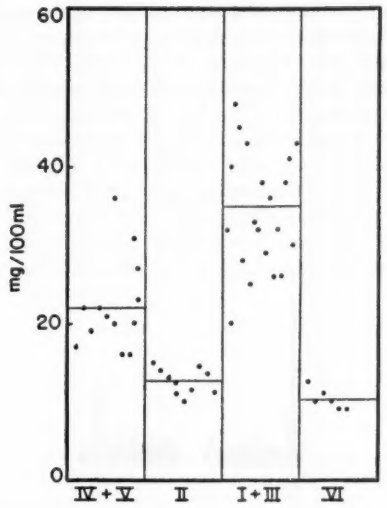
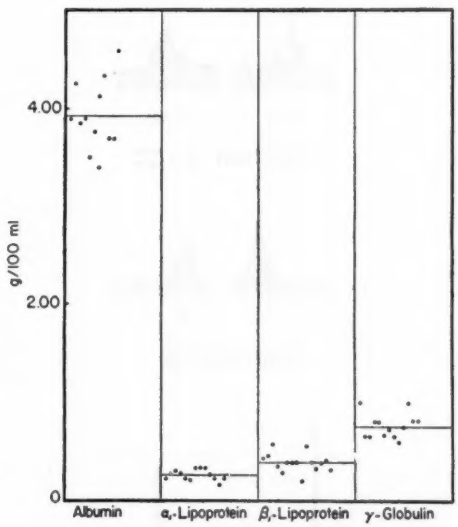


FIG. 4. DISTRIBUTION OF PROTEIN-BOUND CARBOHYDRATE (HEXOSE) AMONG THE FRACTIONS



	% Total Plasma Protein			
Average	56.5	3.8	5.5	10.8
High	65.5	6.0	8.6	14.0
Low	51.1	2.0	3.1	9.3

FIG. 5. CONCENTRATIONS OF SERUM ALBUMINS, α-LIPOPROTEINS, β-LIPOPROTEINS AND γ-GLOBULINS IN PLASMA DETERMINED BY CHEMICAL ANALYSIS OF THE FRACTIONS

exact quantities of hexose present in the complicated group of glycoproteins and mucoproteins of normal human plasma, coupled with the rather general distribution of protein-bound carbohydrate throughout the globulins in plasma (20), has made it impossible to interpret these data in a way similar to the lipid distributions (Figure 5). The data are presented as a useful definition of normal hexose distribution. The hexose in Fraction VI, normally combined principally with an α_1-globulin described by Schmid (21), has been found to show marked increases in certain abnormal plasmas.

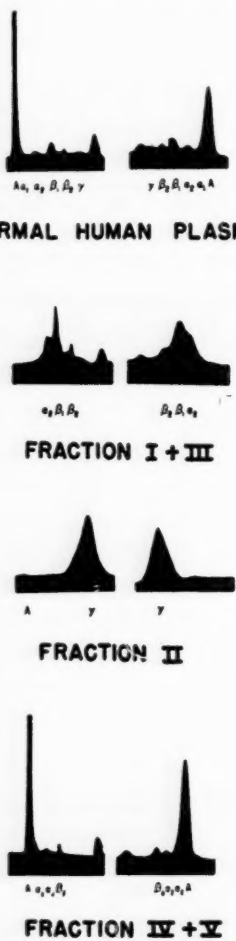


FIG. 6. ELECTROPHORETIC DIAGRAMS OF HUMAN PLASMA AND OF THREE OF THE FOUR PRIMARY FRACTIONS

TABLE II

Distribution of electrophoretic components in Fraction IV + V

	Per cent of fraction						
	Albumin	α_1	α_2	β_1	β_2	Fibrinogen	γ
	85	7	2	5.5	0.5	—	—
	81	6	6	7	—	—	—
	80	10	4.5	5	0.5	—	—
	81	6	7	5	1	—	—
	84	5	4.5	6	0.5	—	—
	84	7	3	5	1	—	—
	82	6	5	6	1	—	—
	79	10	4	6	1	—	—
	84	7	3	6	—	—	—
	78	12	3.5	6	0.5	—	—
	79	8	9	3	1	—	—
	78	11	5	5	1	—	—
Average	81.3	7.8	4.7	5.5	0.7	—	—

Electrophoretic Composition of Fractions

Typical electrophoretic composition data for Fractions IV + V, II, and I + III are presented in Figure 6 and in Tables II, III, and IV, respectively. The values for β_2-globulins in Table II are on the borderline of interpretation, and could have been included with the β_1-peak in the diagrams. Fraction IV + V separated on larger scales (2) was very rarely found to contain β_2-globulins.

Concentration of Serum Albumins, α-Lipoproteins, β-Lipoproteins, γ-Globulins and Fibrinogen

The concentrations of serum albumins, α-lipoproteins, β-lipoproteins, and γ-globulins are shown

TABLE III

Distribution of electrophoretic components in Fraction II

	Per cent of fraction						
	Albumin	α_1	α_2	β_1	β_2	Fibrinogen	γ
	6	—	—	—	4	—	90
	4	—	—	—	4	—	92
	4	—	—	—	4	—	92
	3	—	—	—	2	—	95
	5	—	—	—	3	—	92
	—	—	—	—	4	—	96
	1	—	—	—	3	—	96
	1	—	—	—	1	—	98
	2	1	—	—	5	—	92
	5	—	—	—	8	—	87
Average	3.1	0.1	—	—	3.8	—	93.0

in Figure 5, along with the percentages of the total plasma proteins which they represent. Serum albumins were determined by the hemin-binding method on Fraction IV + V rather than on whole plasma in order to avoid errors due to any nonspecific interference of other proteins.

The quantities of α-lipoproteins and β-lipoproteins accord well with previous estimates (22, 16) based on fractionation studies of large pools of normal human plasma, and with the studies on individual sera reported by Gofman, Lindgren and Elliott (23).

In view of the rather satisfactory electrophoretic (Figure 6, Table III) purity of the γ-globulins in Fraction II, the total protein in Fraction II has been

TABLE IV

Distribution of electrophoretic components in Fraction I+III

	Per cent of fraction					
	Albumin	α_1	α_2	β_1	β_2 + Fibrinogen	γ
	—	—	13	53	27	7
	—	3	9	55	23	10
	—	4	10	50	29	7
	1	1	11	57	28	2
	—	3	14	45	31	7
	—	3	10	58	26	3
	—	5	57		32	6
	—	5	54		36	5
	2	4	24	43	19	8
	1	4	60		31	4
	1	6	29	30	28	6
	2	2	14	50	23	9
	—	5	8	55	21	11
	—	2	9	61	18	10
Average	0.5	3.5	13.3	49.4	26.5	6.8

taken for the present purposes as a measure of the total γ-globulins of plasma. Data to be presented below concerning the distribution of added γ-globulin, along with considerable experience with pathological plasmas in which the γ-globulins were elevated, have confirmed the justification for this approximation.

In some of the early studies, fibrinogen was determined on Fraction I + III by the method employed by Morrison for the study of Fraction I (6). These determinations were discontinued because of extensive occlusion in the clot of the other proteins present in Fraction I + III, in confirmation of the findings of Morrison with Fraction III–0 euglobulin, a fraction also rich in β-lipoproteins (24).[26] From previous studies on citrated plasma in this laboratory (25), using the method of Cullen and Van Slyke (26), a corrected average value of 0.28 g. fibrinogen per 100 ml., or 4.04% of the total plasma proteins may be taken to supplement the data of the present series.

Recovery of Added Plasma Proteins

The recoveries in the separated fraction of added γ-globulins, β-metal combining protein and β-lipoproteins were studied. The γ-globulin preparation employed was prepared on a large scale by Method 10 (2) and was 99% pure by electrophoresis, with albumin as the only impurity detected. The crystalline β-metal combining protein (27) and the purified β-lipoproteins (16) were isolated as previously described. Each protein was added in concentrated solution to one half of a sample of plasma so as to raise the concentration of the constituent to roughly twice the normal, leaving the other half as the control. Table V shows the increase in the various fractions calculated as per cent of the quantity of the constituent added.

The distribution of the added γ-globulins between Fractions II and I + III was as favorable as for normal concentrations. By electrophoretic analysis, γ-globulins remained absent from Fraction IV + V, and, as shown in Table VI, no significant changes occurred in the electrophoretic distribution of Fraction I + III.

[26] A number of determinations of fibrinogen were made on separate samples of the plasma by the method of Morrison (6). The data were corrected on the assumption, based on an extrapolation of the data of Morrison, that occlusion of other proteins amounted to 40% of the weight of the fibrin. The range of values so obtained was 0.15 to 0.43 g. per 100 ml., with an average of 0.30 g. or 4.3% of the total plasma proteins.

TABLE V

Recovery of added plasma proteins

Protein added		Increase in fraction as per cent added constituent		
Nature	Conc.	IV + V	II	I + III
	g./100 ml.			
γ-globulins	0.60	0	93	10
β-metal combining protein	0.25	0	0	0
β-lipoproteins (as cholesterol)	0.39	0	0	92
	0.13	2	−1	93

TABLE VI

Effect of addition of plasma proteins on electrophoretic composition of fractions

Protein added	Fraction analyzed	Per cent of fractions						
			Albumin	α_1	α_2	β_1	β_2 + Fib.	γ
γ-globulins	I+III	Before	1	7	64		25	3
		After	4	6	56		29	4
β-metal comb. protein	IV+V	Before	83	9	3	5	—	—
		After	78	8	4	9	—	—
β-lipoproteins	I+III	Before	—	6	58		30	6
		After	—	5	66		21	8

The β-metal combining protein, due to the small amount added, failed to cause a change in the gross distribution of the proteins among the fractions (Table V). However, the electrophoretic data showed clearly the marked increase in β_1-globulins in Fraction IV + V (Table VI), whereas no changes were observed in the other fractions.

The recovery of β-lipoproteins in Fraction I + III, and the failure to contaminate the α-lipoproteins in Fraction IV + V, were striking. Table VI shows the absence of any significant increase in the small quantity of γ-globulins present in Fraction I + III.

These recovery experiments confirmed the selectivity of the separations, and showed that considerable increases in at least certain of the plasma proteins did not derange the system of fractionation. It was particularly important that large variations in the proportions of the strongly interacting components, γ-globulins and β-lipoproteins, could be tolerated in the separation of Fraction I + II + III and the subsequent extraction of Fraction II.

SUMMARY

1. Method 10 of plasma fractionation developed by Cohn and his co-workers has been adapted for the quantitative estimation of several protein and lipoprotein components in small quantities of normal human plasma.

2. In the system of analysis presented in this communication samples of 5 ml. of plasma were separated by filtration into four fractions and the amounts of albumin, γ-globulins, α-lipoproteins and β-lipoproteins were determined. In addition, the distributions of cholesterol and phospholipid and of protein-bound carbohydrate (hexose) among the fractions were studied.

3. The proteins are present in the various fractions in an unaltered state. Their biologic activities are unimpaired and can be measured.

4. Of interest is the concentration in Fraction II of more than 90% of the γ-globulins of the plasma in a purity exceeding 90% by electrophoretic measurements. The other three fractions contain mixtures of unrelated proteins.

5. The plasma lipoproteins are divided into two primary groups; the α-lipoproteins in Fraction IV + V contain a considerably higher proportion of phospholipids to cholesterol than the β-lipoproteins in Fraction I + III.

6. Added amounts of various pure proteins were in each case recovered in the appropriate fractions, proving that even large variations in the proportions of the various proteins do not interfere with the system of fractionation.

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PROTEOLYTIC ENZYMES AND PLATELETS IN RELATION TO BLOOD COAGULATION [1]

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Ferguson's (1, 2) concept of a role for proteolytic enzymes (3) in the mechanisms of blood clotting was that of a weak digestive action "disaggregating" (Pope, 1938) the complex unions of plasma proteins with calcium and thromboplastic phospholipid (probably cephalin), thereby mobilizing or making available these activators and thus, in a sense, "catalyzing" the prothrombin activation (4). Observations believed to support such a theory included: *1*) the clot-aiding effects, particularly referable to the conversion of prothrombin into active thrombin, on adding (crystalline) trypsin (*2*, 4); and *2*) the evidence, not altogether conclusive (4) nor confirmed (5–7), that preparations of serum protease (tryptase, plasmin, or fibrinolysin) resemble pancreatic trypsin in many ways although differing significantly in others (3).

The establishment of crucial data in this, as in all work on the problem of the conversion of prothrombin to thrombin, is technically difficult because of the biochemical complexities, the presence in the natural clotting system (*e.g.* plasma) of unrecognized factors (some of which have recently been revealed), and questions as to contaminants (the known ones being demonstrable) in even the purest of the isolated reagents available to date (4). From the start (1939) of our work with the enzyme problem we have accepted the current, and still valid, working hypothesis that, under all ordinary ("physiological") circumstances, prothrombin requires for its activation free Ca-ions and a phospholipid-containing material for which the terms "thromboplastin" or "thrombokinase" are commonly used. It has more recently been shown by a number of workers that other factors are also involved and evidently must have been unwittingly provided in the test materials of all early experiments. Our own observations (8) that, with very carefully purified and treated reagents, no thrombin could be detected in over 24 hours in mixtures of prothrombin, brain thromboplastin, and calcium salt, but required the addition of another factor or factors which could be supplied in the form of Ware and Seegers' (9) "serum accelerator globulin" (AcG) preparation, caused us to add this reagent to the purified test systems in the present studies. Under these circumstances, as in the past, we feel that our studies are concerned primarily with the basic "thromboplastic" mechanisms.

The present work commences with the confirmation of the clot-aiding effects of trypsin and of serum fibrinolysin on ordinary plasma and supplements this with an experiment on platelet-free plasma. Proceeding thence to the study of thrombin formation from highly purified prothrombin (with added AcG), a number of enzyme preparations, activators, and inhibitors have been tested, in various combinations, in an effort to shed further light upon the possible mechanisms of action of the proteolytic enzymes in such clotting systems. The thromboplastic role of platelets in relation to these phenomena has been given special attention. The weight of evidence eventually leads to the conclusion that whereas trypsin does seem to act with parallelism of its clot-aiding and proteolytic effects, serum fibrinolysin can still aid thrombin formation (in these systems) even when the proteolytic action is reduced greatly or abolished by appropriate enzyme inhibitors. This must suggest another factor in the serum enzyme preparation, the possible nature and mode of action of which are investigated and discussed.

MATERIALS

1. Borate *buffer* solution, pH = 7.75, specific resistance 170 ohms (at 21°C), effective ionic strength 0.055, contained (per liter): 11.25 gm. H_3BO_3, 4 gm. $Na_2B_4O_7$, 12 H_2O, 2.25 gm. NaCl.
2. *Plasma*, oxalated or citrated, was prepared from dog or beef blood. In special studies, cited in the text,

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(*a*) "deplateletized" plasma (PFP) was prepared by the silicone technic (10) and long centrifugation (multispeed) in the refrigerated centrifuge; (*b*) "defibrinated" plasma (FFP) was obtained by heating at 51°C for one-half hour and centrifugation; (*c*) "prothrombin-free" plasma (S.Pl.) was obtained by five successive filtrations through Seitz pads, according to the method of Owren (11).

3. *Serum,* obtained from beef blood, was rendered prothrombin-free by adsorption with $BaSO_4$ (12).
4. *Fibrinogen:* (F_1) 0.5–1.0% solution in borate buffer of Armour's bovine fibrinogen (Fraction I). This was free from fibrinolysin but contained small traces of prothrombin which, for many tests, was removed by $BaSO_4$ adsorption (F_2).
5. Purified *prothrombin* (Pro), bovine, preparations of Dr. W. H. Seegers (Wayne University), stock solution (0.1%) in borate buffer. The solutions used in most of the cited experiments contained an insignificant trace of thrombin (clotting times with $F_2 > 30$ min.) and some "accelerator" factor(s), but the latter was always supplemented with added AcG.
6. *Accelerator globulin* (AcG), bovine "serum type," preparation of Drs. A. G. Ware and W. H. Seegers, stock solution (0.1%) in borate buffer. It contained traces of prothrombin and a minute amount of thrombin, considered non-significant in the dilutions employed for the cited experiments.
7. *Thromboplastins:* Tpln. A, a highly active thromboplastin prepared from acetone-dried dog brain; Tpln. B, Schieffelin's stabilized (horse brain) thromboplastin, courtesy Drs. E. W. Blanchard and C. F. Gerber.
8. *Fibrinolysin* (Lysin): purified preparation from beef serum, courtesy Dr. E. C. Loomis (Parke Davis Co., Detroit). 1% stock solution freshly prepared immediately before use.
9. *Antifibrinolysin* (AFln): purified bovine preparation, courtesy Dr. Loomis.
10. *Trypsin* (pancreatic): (Tryp.), commercial preparation (Fairchild Bros., Foster, N. Y.); *Crystalline trypsin* (XT), courtesy Dr. M. Kunitz (Rockefeller Institute, Princeton).
11. *Soybean inhibitor* (antiprotease): crystalline preparation (SBI), courtesy Dr. Kunitz.
12. *Pancreatic inhibitor* (antiprotease) (PI): Sharp and Dohme (Lot P. A–97–1), courtesy Dr. L. A. Kazal.
13. *Washed platelets* (Plat.): 100 ml. dog blood collected into 13 ml. 3.4% sod. citrate. Repeated centrifuging for a few minutes at 1000 r.p.m. until red cells no longer visible in sediment. Platelets then recovered by one-half hour centrifuging at 5000 r.p.m., resuspended and well washed three times by thorough mixing with successive 50 ml. saline (containing ⅛ vol. 3.4% citrate) and a fourth washing in plain 0.9% NaCl. Final sediment suspended in 1 ml. saline gave a concentrated preparation, Wright-stained smears of which showed only granular platelet material with no leukocytes and only a rare erythrocyte. Grinding the platelet sediment with distilled water on a number of occasions did not modify its behavior in our test systems. The thoroughness of the washing is believed satisfactory for the virtual absence of plasma contaminants.
14. *Thrombin* (Thr.): bovine thrombin (Upjohn's), courtesy Dr. J. T. Correll. Stock solution in borate buffer (100 u./ml.), diluted as required. For the tests of Table VII a special preparation was made and used as described in the text.

Effects of Fibrinolysin on Clotting of Recalcified Plasma

In numerous experiments on oxalated plasma (dog: beef), optimally recalcified, Loomis' fibrinolysin consistently reduced the clotting-times, *e.g.* beef plasma (26°C): *1*) control, 330 sec.; *2*) shorter clotting-times, with increasing lysin, to 135 sec. "optimum" at 1.5–2.0 mg./ml. lysin; *3*) lessening effects with further amounts of enzyme, *e.g.* 205 sec. at 6.0 mg./ml. In the last test, the clot lysed in one-half hour, whereas at 1.5–2.0 mg./ml. (optimal for clotting), fibrinolysis was incomplete in 48 hours at room temperature. That the inhibitory effect of plasma antilysin is responsible is evident from clot-lysis times for any single concentration of lysin tested in *a*) plasma clots, *b*) pure fibrin clots—see Table I.

Effects of Proteases, etc. on Clotting of Recalcified Deplateletized *Plasma*

Deplateletized citrated dog plasma (PFP: see Materials) was used for the tests of Table I. Triplicate clotting tests immediately after preparation of PFP, using 0.9% NaCl diluent and simple recalcification at room temperature, showed no clotting until 32–36 hours. The marked clot-accelerating effects of 4 mg. Loomis' fibrinolysin, of 25–100 μg. crystalline trypsin, and of a diluted (suboptimal) brain thromboplastin, are evident. Five-tenths mg. soybean inhibitor reverses the clot acceleration in all cases, but particularly in the case of trypsin.

In these and other experiments we have made with deplateletized plasmas, clotting in times exceeding half an hour appeared to involve some unexplained questions of non-specific factors relating to colloidal stability, rather than specific ("thromboplastic") factors, like those in the data of Table I, which have a very marked influence in reducing the clotting time.

As in all plasma experiments, fibrinolytic effects are weak because of natural antilysin and prob-

TABLE I

Effects of proteases, etc. on clotting times of platelet-poor plasma

Clotting times and lysis times, at 38°C, of 0.5 ml. citrated dog plasma (platelet-poor: silicone technic), recalcified with 0.1 ml. M/10 $CaCl_2$, in presence of 1.0 ml. additional volume of borate buffer (pH = 7.75) containing the amounts of other reagents noted. ∞ = no lysis in 10 days.

	Inhibitor	Activator	Clotting time	Lysis-time	
				Plasma clot	Std. fibrin clot (13)
1.	0	0	2000 sec.	∞	
2.	SBI (0.5 mg.)	0	2880 sec.	∞	
3.	0	Lysin (4 mg.)	127 sec.	5 days (tr.)	5½ min.
4.	SBI (0.5 mg.)	Lysin (4 mg.)	525 sec.	∞	1½–2 days
5.	0	XT (tryp.) (25μg.)	365 sec.	4–5 days	9 min.
6.	SBI (0.5 mg.)	XT (tryp.) (25μg.)	2490 sec.	∞	5 days
7.	0	XT (tryp.) (50μg.)	168 sec.	2–3 days	5½ min.
8.	SBI (0.5 mg.)	XT (tryp.) (50μg.)	2400 sec.	∞	5 days
9.	0	XT (tryp.) (100μg.)	118 sec.	18 hours	3 min.
10.	SBI (0.5 mg.)	XT (tryp.) (100μg.)	1800 sec.	∞	4½ days
11.	0	Tpln. B (dil.)	65 sec.	∞	
12.	SBI (0.5 mg.)	Tpln. B (dil.)	352 sec.	∞	

ably also, in part, due to instability of the enzyme preparations over the long periods (days). The standard clot (fibrin) lysis times included in the last column of Table I afford a reliable measure of respective lytic potencies (13).

Effect of Fibrinolysin on Conversion to Thrombin of Prothrombin in Fibrinogen-Free Plasma

In several experiments, beef plasma heated to 51°C for one-half hour and centrifuged from the coagulated protein was obtained fibrinogen-free, by tests with purified thrombin. Table II shows, by means of the shortening clotting times of successive samples removed after the stated incubation periods and tested on fibrinogen, the progressive thrombin formation in such fibrinogen-free plasma after recalcification, with and without fibrinolysin added. The evident shortening of the time required to reach the best (*i.e.* quickest) clotting time and the fact that this is faster (12 sec.) with the lysin present than in the control (21 sec.), show that the enzyme acts by facilitating thrombin formation. Any tissue thromboplastin will give similar results.

Effects of Fibrinolysin and Other Activators on Thrombin Formation from Purified Prothrombin

Table III shows, by means of clotting times (sec.) of samples of various thrombic mixtures added to test fibrinogen after the stated incubation periods, the *relative* amounts of thrombin formation from a purified prothrombin solution. We have presented a critical analysis of this method in a recent publication (4). In brief, the shortest clotting time reached measures "complete" conversion of prothrombin to thrombin under the particular test conditions, while the incubation

TABLE II

"Thromboplastic" action of fibrinolysin in activation of prothrombin in recalcified fibrinogen-free plasma

Two and five-tenths ml. plasma (beef: defibrinated at 51°C for one-half hr.), recalcified with 0.25 ml. M/10 $CaCl_2$, with (*1*) 0.75 ml. borate buffer (control) or (*2*) 0.75 ml. 1% lysin. After stated incubation periods, 0.25 ml. samples tested with 0.5 ml. 0.5% fibrinogen (F_1), noting clotting times (sec.), at 26°C, pH = 7.75.

	Added agent	Incubation period (*min.*)											
		1′	2′	3′	4′	5′	6′	7′	8′	10′	11′	15′	20′
1.	Buffer (only)	200″	190″	150″	107″	60″	—	28″	24″	21″	—	21″	24″
2.	Lysin	100″	50″	19″	14″	12″	12″	—	—	—	12″	—	—

period needed for this is a measure of the effectiveness of the activator factors. Since calcium is optimal and a sufficiency of other known accelerators is supplied in the AcG added (perhaps presumptively), the "thromboplastic" factor is believed to be the significant variable. We have not included in the present work any attempt to define a thrombin "unit" or "percentage" (of prothrombin activated), since mere inspection of the data in each experiment suffices clearly to bring out the essential points of comparison. Thus, in the experiments of Table III: *1*) No clots in one-half hour shows inadequacy of calcium and AcG alone to activate the purified prothrombin solution; *2*) fibrinolysin, in addition, has so little effect as to be practically negligible; *3*) platelets, on the other hand, contribute a definite "thromboplastic" effect and result in a fairly satisfactory thrombin formation, although inferior to a typical potent tissue thromboplastin (tests 5). The fact that lysin and platelets, acting in conjunction (tests 4), produce the best activation, though not yielding more thrombin (10 sec. clotting time optimum) than in tests 5, argues strongly for a "platelet thromboplastin potentiating" (PTP) action of the enzyme.

TABLE III

Effects of fibrinolysin and other activators on thrombin formation from purified prothrombin

Clotting times (sec.), at 26°C, pH = 7.75 (borate buffer), for 0.5 ml. F_1 + 0.25 ml. thrombic mixture (after stated incubation periods), containing 0.2 ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.5 ml. M/20 $CaCl_2$, and other activators in amounts specified, with borate buffer solution to total volume 5 ml. ∞ = No clot in one-half hr.

	Activators	Incubation period (*min.*)						
		1′	3′	5′	10′	20′	40′	60′
1.	0	—	—	∞	∞	∞	∞	∞
2.	Lysin (3 mg.)	—	—	390″	380″	368″	335″	320″
3.	Plat. (0.5 ml.)	420″	155″	60″	21″	—	—	18″
4.	Lysin (3 mg.) Plat. (0.5 ml.)	75″	22″	16″	11″	11″	—	10″
5.	Tpln. A (0.5 ml.)	240″	120″	10″	10″	10″	10″	10″

Clot-Aiding Effects of Platelets

The designation of the mode of action of platelets as "thromboplastic" in the preceding section would not only be in line with the definition and working hypothesis presented in the introduction, but the presence of added AcG (ineffective, with Ca alone) would argue against the platelet activators being significantly of the type supplied by serum accelerator globulin. Since a paper by Ware, Fahey and Seegers (14), appearing while the present studies were in progress, claimed that platelets supplied very little thromboplastin, but significant amounts of AcG, and some factor (probably of the type we call "fibrinoplastic" [15]) which aids the thrombin-fibrinogen reaction, these claims were carefully and repeatedly investigated with our platelet preparations.

TABLE IV

Effect of washed platelets as an "accelerator" of the activation of AcG-poor prothrombin

Two and five-tenths ml. vol. "thrombic mixtures" (T) containing borate buffer (pH = 7.75), 0.1 ml. Pro. (0.1%), 0.25 ml. $CaCl_2$ (M/20), and activators noted. Clotting times (sec.) of 0.5 ml. F_2 + 0.25 ml. T (at stated incubation periods): 26°C

	Activators *ml.* (dilution)	Incubation time (*min.*)				
		5′	10′	20′	40′	60′
1.	Tpln., 0.25	420″	300″	202″	141″	110″
2.	Tpln., 0.25 Plat., 0.1	390″	240″	80″	22.4″	15″
3.	Tpln., 0.25 AcG, 0.1 (0.001%)	300″	140″	52″	25.8″	20.3″
4.	Tpln., 0.25 AcG, 0.1 (0.1%)	11.4″	9.4″	8.0″	7.8″	7.6″

Table IV is a typical experiment to illustrate how little "AcG-like" action was found in these well-washed platelet preparations. The poor thrombin formation in tests 1 indicates the deficiency of AcG in the chosen prothrombin, so that optimal calcium and tissue thromboplastin are insufficient activators in the hour incubation period. Compare the excellent activation in 4, with plenty of AcG added. Platelets (tests 2) produced relatively little effect, roughly comparable to the weaker AcG addition in 3, which amounts to a concentration of only 1:2,500,000 in the final thrombic mixture.

Tested on thrombin-fibrinogen mixtures, our platelets showed, if anything, a few seconds' prolongation of clotting times and no "fibrinoplastic" enhancement of clotting at several dilutions. We did find some evidence that a commonly encountered instability of thrombin solutions was apt to be less in the presence of platelets, however.

Effects of Varying *Fibrinolysin Concentrations (Platelets Constant) and of* Varying *Platelet Concentrations (Fibrinolysin Constant) on Activation of Purified Prothrombin*

These experiments were performed by our usual technic of following the activation of prothrombin by means of the clotting times for fibrinogen (F_2) of samples of the various thrombic mixtures, removed after stated incubation periods. The 2.5 ml. mixtures contained 0.1 ml. Pro. (0.1%), 0.25 ml. $CaCl_2$ (50mM), 0.1 ml. AcG (0.1%), and the activators studied. From the series of data obtained, only the clotting-time values *at a single incubation period* were selected for comparing the effects of varying amounts of the fibrinolysin or platelet activators, and the accompanying graphs are plotted in this way.

In Figure 1 the three curves A, B, C are, respectively, the clotting times (sec.) of samples tested after one, five, and 60 min. incubation. The mixtures all contained 0.1 ml. platelet preparation but the fibrinolysin concentration was varied and is expressed as mg. per 2.5 ml. of the mixture. Curve B^1 is from another experiment and, therefore, not quantitatively comparable with B, but,

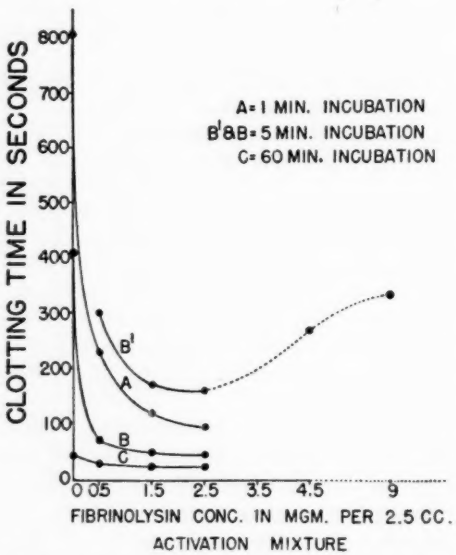

FIG. 1. EFFECT OF VARYING FIBRINOLYSIN CONCENTRATION WITH PLATELETS CONSTANT

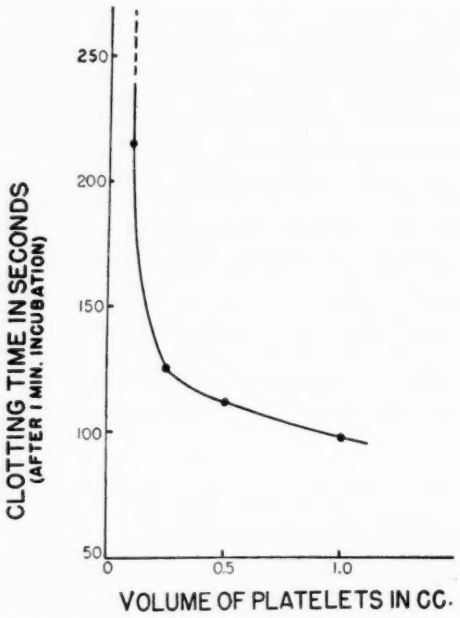

FIG. 2. EFFECT OF VARYING PLATELET CONCENTRATION WITH FIBRINOLYSIN CONSTANT

by extending the lysin concentration up to 9 mg. it brings out the additional point of an "optimum" for the enzyme effect at about 2.5 mg. (per 2.5 ml.) in this particular experiment. The data clearly show that the "potentiating" effect of fibrinolysin is more marked on the earlier phases of prothrombin activation and does depend upon the enzyme concentration, but, according to the 60 min. data of Figure 1, C, does not significantly influence the final thrombin yield (shortest clotting times).

Figure 2 is an experiment with fixed and approximately optimal fibrinolysin (2.5 mg./2.5 ml.) but varying the amount of platelet preparation between 0 and 1.0 ml./2.5 ml. of thrombic mixture. The one min. incubation samples are selected for the clotting-time test data plotted.

Figure 3 shows controls (five min. and 60 min. incubation samples) in an experiment with varying platelets (volumes plotted logarithmically) and no fibrinolysin present. The clotting times are significantly longer than when lysin is added and are expressed in minutes.

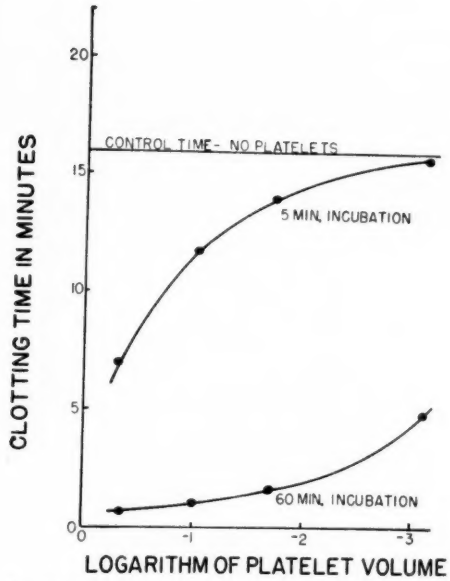

FIG. 3. EFFECT OF VARYING PLATELET CONCENTRATION; NO FIBRINOLYSIN PRESENT

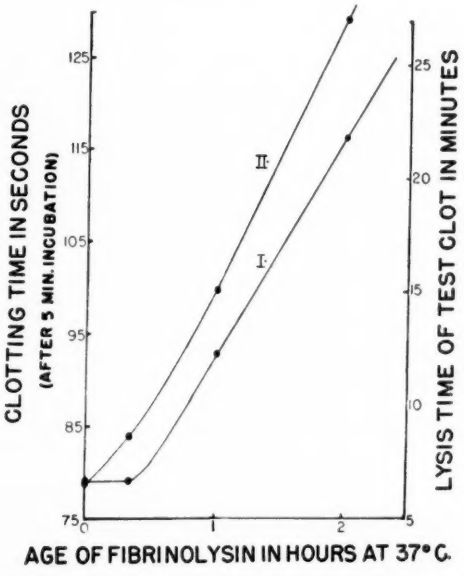

FIG. 4. LOSS OF THROMBOPLASTIC (I) AND LYTIC (II) ACTIVITY OF PROTEASE

Effects of Thermal Instability of Fibrinolysin on its Lytic Potency and on its Platelet Thromboplastin Potentiating Effects

Loomis' fibrinolysin (1% solution in borate buffer) was allowed to undergo deterioration at 37°C (water bath) and samples, removed at stated intervals of aging, tested simultaneously for *1*) fibrinolytic potency, by the method of Lewis and Ferguson (13), and *2*) platelet thromboplastin potentiating effect, in tests closely similar to those described above. Figure 4 plots, against age of lysin (up to two hours at 37°C), *I.* fibrinolysis times, which lengthen with weakening enzyme potency, and *II.* clotting times of five min. incubation samples, which, according to data of the preceding section, give a relative measure of "thromboplastic" potency. It would appear that the two activities of the enzyme preparation weaken at very similar rates. If the clotting-time data are plotted against residual fibrinolytic activity (*units* computed from a reference curve [13]), the figure will resemble the descending portion of curves B and B' of Figure 1, in which dilution, instead of deterioration, was the method of reducing the enzyme activity.

Effects of Protease-Inhibitors on Conversion of Prothrombin to Thrombin, in Presence of Brain Thromboplastin, Platelets, and Fibrinolysin

Preliminary to these studies, extensive experiments were carried out clearly to establish that *1*) none of the antienzymes inhibited the thrombin-fibrinogen reaction, *2*) fibrinolysin (0.1%) had little, if any, effect on strong thrombin (> 1 u./ml.) but could lengthen clotting times with weak thrombins (< 0.1 u./ml.) due to the opportunity afforded for *fibrinogen*olysis (*cf.* [4]).

Proceeding to the testing of thrombin formation from purified prothrombin with added AcG and optimal calcium and other reagents stated, the data of Table V record a typical series of experimental results. Interpreting the clotting-time tests in the usual way, the results indicate most significantly that pancreatic inhibitor (PI) and serum antifibrinolysin (AFln) are *not* inhibitory to the cited thromboplastic agents (tpln. A or plat.-lysin mixtures) even though they prevented clot lysis in the fibrinolysin-containing tubes

TABLE V

Effects of antiproteases on thromboplastic agents

One-tenth ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.25 ml. Ca (M/20), activators and inhibitors stated, and borate buffer to 2.5 ml. vol. Clotting times (sec.) of 0.25 ml. mixture + 0.5 ml F_1, at stated incubation times, 25°C.

	Activators *ml.* (conc.)	Inhibitors *ml.* (conc.)	Incubation time (*min.*)				
			5′	10′	20′	40′	60′
1.	Tpln. A, 0.25	0	22.5″	17.1″	15.2″	15.0″	14.2″
2.	Tpln. A, 0.25	SBI, 0.1 (0.01%)	450″	420″	450″	450″	380″
3.	Plat., 0.1 Lysin, 0.2 (0.5%)	0	49″	25.2″	21.8″	21.3″	21″
4.	Plat., 0.1 Lysin, 0.2 (0.5%)	SBI, 0.1 (0.1%)	630″	135″	42″	38.5″	35.4″
5.	Tpln. A, 0.25	0	25.5″	21.1″	19.2″	18.4″	19.6″
6.	Tpln. A, 0.25	PI, 0.1 (0.1%)	28.8″	23.3″	21.8″	21.5″	20″
7.	Plat., 0.1 Lysin, 0.15 (1%)	0	29.1″	23″	22″	22″	22″
8.	Plat., 0.1 Lysin, 0.15 (1%)	PI, 0.1 (0.1%)	28″	22.5″	19.9″	20″	20″
9.	Tpln. A, 0.25	0	22.5″	15.4″	14.6″	13.1″	12.2″
10.	Tpln. A, 0.25	*AFln, 0.15 (0.5%)	28.5″	22.4″	20.6″	21.2″	21″
11.	Plat., 0.1 Lysin, 0.15 (1%)	0	58.2″	29.3″	24.6″	24.3″	25″
12.	Plat., 0.1 Lysin, 0.15 (1%)	*AFln, 0.15 (0.5%)	37.6″	24.9″	22.4″	22.2″	20″

* A 15 min. incubation period, at 26°C, of antifibrinolysin with thromboplastic agent, was used to permit development of its antienzyme effect (13).

whereas inhibitor-free controls all lysed within 12–24 hours. Soybean inhibitor (SBI) was exceptional in inhibiting the brain thromboplastin (tests 2), so that it evidently has some "antithromboplastic" action, despite which its effect on activation with plat.-lysin (tests 4) is strongly inhibitory only in the earlier activation phases.

Effects of Antiproteases on Thromboplastic Action of Trypsin-Platelet Mixtures

Similar experiments to those in the preceding section were performed with pancreatic trypsin (tryp.) instead of fibrinolysin, and with the antiproteases PI and AFln previously used. The results are given in Table VI. Here platelets alone (tests 1) and trypsin alone (tests 2) gave very slow and inadequate activation of the prothrombin-AcG-Ca mixture, whereas plat. + tryp. (tests 3) exerted a marked thromboplastic effect on the thrombin formation. In contrast to the previous results with fibrinolysin, the trypsin synergism with platelets was apparently completely blocked by both the tested inhibitors, PI (tests 4) and AFln (tests 5).

TABLE VI

Effects of antiproteases on thromboplastic action of trypsin-platelet mixtures

Two-tenths ml. Pro. (0.1%), 0.2 ml. AcG (0.1%), 0.5 ml. Ca (M/20), activators and inhibitors stated, and borate buffer to 5 ml. vol. Clotting times (sec.) for 0.25 mixture + 0.5 F_1 at stated incubation periods (25°C).

	Activators *ml.* (conc.)	Inhibitors *ml.* (conc.)	Incubation time (*min.*)				
			5′	10′	20′	40′	60′
1.	Plat., 0.1	0	720″	540″	270″	100″	65″
2.	Tryp., 0.3 (0.01%)	0	182″	140″	95″	58″	51″
3.	Plat., 0.1 Tryp., 0.3 (0.01%)	0	47″	29.5″	26.5″	23.5″	23.2″
4.	Plat., 0.1 Tryp., 0.3 (0.01%)	PI, 0.1 (0.1%)	660″	240″	175″	113″	96″
5.	Plat., 0.1 Tryp., 0.3 (0.01%)	AFln, 0.3 (4%)	710″	490″	205″	103″	71″

"Thromboplastic" Tests with Enzymes (sans Platelets) and Anti-Enzymes

Numerous tests on prothrombin, AcG, calcium mixtures, with fibrinolysin and with trypsin, but without any platelet or other added thromboplastin, merely confirmed the all but negligible "thromboplastic" effects of the enzymes alone. None of the inhibitors (SBI, PI, AFln), acting alone, have significant effects except for some inhibition of the very minor thrombin formation (seen in controls) by SBI. The differences with enzyme + inhibitor combinations were slight and chiefly a matter of failure to inhibit the minor actions of fibrinolysin with PI and AFln, and the blocking of minor actions of 0.01% trypsin. There is no need to cite these experimental data in detail.

Is There Thrombin in the Fibrinolysin Preparation?

It is extremely difficult to prepare fibrinolysin without significant contamination with thrombin. Loomis' serum enzyme preparation was selected for the present studies particularly because it appeared to fulfill the claim (16) to be free from thrombin and prothrombin. Owing to fibrinogenolysis, however, it is difficult to put this to an exacting test, but we sought to overcome this difficulty by neutralizing 0.3 ml. 1% lysin with 0.3 ml. 0.5% antilysin (Loomis') and testing on recalcified prothrombin-free fibrinogen (F_2). Even this test was inconclusive, however, as a small clot did appear in about two hours, persisting for at least four days (at 26°C). The antilysin alone caused no clot.

Effects of Very Weak Thrombin on the Thromboplastic Action of Platelet Preparations

We have repeatedly confirmed the ability to secure much better thrombin formation in purified prothrombins treated with calcium, AcG, and platelets, when a tiny trace of thrombin was added, far too little merely to add its own clotting-time contribution.

A special test of this phenomenon was made with the purest thrombin available to us. This reagent was obtained by allowing a 0.1% borate buffer solution of one of Seegers' highly purified prothrombins to activate for several weeks in the refrigerator (4°C) with added calcium salt only.

TABLE VII

Effects of weak thrombin on platelet thromboplastin

One-tenth ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.25 ml. Ca (M/20), activators cited, with borate buffer to 2.5 ml. vol. Clotting times (sec.) for 0.25 mixture + 0.5 ml. F_1, at stated incubation times (26°C).

	Activators (*ml.*)	Incubation time (*min.*)				
		5′	10′	20′	40′	60′
0.	0	885″	913″	920″	970″	1010″
1.	Plat. (0.1)	810″	620″	330″	190″	97″
2.	Plat. (0.1) Thr. (0.25)	145″	52″	22″	12″	9″
3.	Plat. (0.1) 0.5% lysin (0.3)	60″	46″	15″	12″	9″

At the end of this time it had formed a potent thrombin, the activity of which was not increased by incubation with thromboplastin, indicating that unaltered prothrombin was no longer present. Several dilutions of the lysin- and thromboplastin-free product were examined for the platelet thromboplastin potentiating action. In tests 2 of Table VII an effect, qualitatively and approximately quantitatively similar to that obtained with 0.3 mg. fibrinolysin (tests 3), is noted. When freshly prepared, this particular thrombin dilution clotted the test fibrinogen in 750 seconds, but its potency decreased fairly rapidly on standing. Owing to this thrombin instability, exact control tests, *minus* the platelets, were impracticable. Such tests were attempted, however, and did show, in a reasonably satisfactory manner, that the thrombin itself caused only insignificant shortening of the control (tests 1) clotting times.

Attempts at Isolation from Prothrombin-Free Plasma of a Factor Which Potentiates the Thromboplastic Effect of Platelets

Oxalated beef plasma was passed five times through Seitz filter pads to render it prothrombin-free by the method of Owren (11). Tests for thrombin and prothrombin being entirely negative, the Seitz plasma (S.Pl.) was then subjected to successive fractionation at (*a*) 20, (*b*) 30, (*c*) 40, and (*d*) 50% saturation with ammonium sulfate, at room temperature. All precipitates (redissolved in saline) and (*e*) the final supernatant ("albumin") were dialyzed until sulfate-free.

Tests on dialysates: with the usual prothrombin,

AcG, Ca system, with platelets, all proved negative. The fractions were then shaken with chloroform, following the old method (17, 18) for activating fibrinolysin.

Tests on $CHCl_3$-treated dialysates: the usual testing for potentiation of platelet thromboplastin was again carried out, with the result that the only significant acceleration of thrombin conversion was found in Fraction C (30–40% $[NH_4]_2SO_4$).

Control tests: I. $CHCl_3$-treated Fraction C, in same dilution as in preceding tests, showed (*a*) traces of thrombin, *e.g.*, 0.25 ml. C(dil.) + 0.5 ml. F_2: clotting time (26°C) 575 sec.; (*b*) some fibrinolytic action, *e.g.*, lysis of foregoing clot in about 12 hours. II. The other $CHCl_3$-treated fractions showed no evidence of thrombin or lysin.

In view of the data of the preceding section (Table VII), the trace of thrombin in $CHCl_3$-treated Fraction C, as well as the fibrinolysin, and possibly other possible factors remaining to be detected, all need critical evaluation.

It was surprising to find any thrombin at all in the above material. Since $CHCl_3$ is known to inactivate antithrombin, we performed some tests which showed a considerable reactivation of thrombin which had lost practically all its activity after incubating 24 hours at room temperature with (*a*) Seitz-filtered plasma (S.Pl.) or with (*b*) $BaSO_4$-adsorbed serum. A thrombin-destroying action of $CHCl_3$, seen also with thrombin solution alone, partly (at least) explains the *partial* recovery. It was found, incidentally, that pancreatic inhibitor retards the antithrombic action and permits the subsequent $CHCl_3$ treatment to give bettter thrombin recoveries.

DISCUSSION

The present investigations confirm earlier observations that both trypsin and serum fibrinolysin (plasmin or tryptase) exert significant clot-aiding effects attributable to participation in the mechanisms of conversion of prothrombin to active thrombin. With purified prothrombin, optimal calcium, and such accessory factors as are supplied with added "serum accelerator globulin," but no added thromboplastin (cell- or tissue-factor), these enzyme preparations have little, if any, "thromboplastic" action of their own, the clotting times of the thrombic mixtures plus purified fibrinogen solution being too long to give significant results. In previous studies (4) with potent tissue thromboplastins, clotting times in control tests, without enzyme, were too short to give more than suggestive evidence that addition of these proteases could "potentiate" the thromboplastic action, particularly in its earlier phases. In the present studies, well-washed platelet preparations, shown to be without significant effects other than those of a relatively weak "thromboplastic" character, were markedly potentiated by the enzyme materials in question.

This PTP ("platelet thromboplastin potentiating") effect of the serum fibrinolysin parallels its lytic activity both in dilution experiments and during deterioration at 37°C. However, protease inhibitors from pancreas (PI) and from serum (AFln) do not abolish the PTP effect, although they do inhibit the enzymic fibrinolysis.

Soybean inhibitor (SBI) has additional "antithromboplastic" effects, even for tissue- and platelet-thromboplastins without proteolytic enzyme (Table V), so that its effect of at least partly inhibiting the PTP action may very well be due to its antithromboplastic instead of its antilytic effects.

In the case of trypsin, however, all the inhibitors abolish the two effects simultaneously. Thus, the arguments we have previously (4) built up for a "thromboplastic enzyme" mechanism accessory to the thrombin-forming phase of blood clotting, are now still valid only in the artificial test systems with pancreatic trypsin. Failure of correlation of the two effects in tests with appropriate inhibitors (serum antifibrinolysin especially) overthrows a large amount of evidence otherwise strongly suggesting a similar role for the natural serum protease fibrinolysin. The conclusion must be that some other factor must be sought for in the enzyme preparation to account for its thromboplastin potentiating effects.

The possibility that this may be a trace of thrombin is perhaps suggested by the discovery (Table VII) of a significant "PTP" effect of very small amounts of thrombin such as may frequently be found as contaminants of serum enzyme and many other blood preparations. However, the extremely minute and possibly doubtful traces of thrombin demonstrable in some special tests with the present lysin, would appear insufficient to invoke this explanation here. Nor is it probable that the minute traces of thrombin, present or appearing in the prothrombin-AcG mixtures (which are very dilute), can be implicated, particularly to explain the sharp *increase* in "thromboplastic" action when the enzyme-platelet-activator is used and

compared with control tests. It is barely possible, however, that these sources of thrombin trace-contaminant may require some consideration in explaining (fully) the thromboplastic action of platelets alone (*i.e.*, without enzyme) as seen in the data of Tables III and IV.

Ware, Fahey, and Seegers (14) found some thrombin formation in platelet-activated mixtures containing stronger prothrombin (330 u./ml.) but none in an hour's incubation with much lower (1.34 u./ml.) prothrombin. If their prothrombin contained the usual trace of thrombin contaminant, these results at the higher concentrations would appear similar to ours, whereas at the lower concentrations the thrombin contaminant may very well have been diluted to insignificance.

Our preliminary attempts to prepare a "platelet thromboplastin potentiating" factor, from apparently thrombin- and prothrombin-free Seitz filtered ($\times 5$) plasma, succeeded only in the case of a $CHCl_3$-treated 30–40% saturated $(NH_4)_2SO_4$ Fraction C, in which definite traces of both fibrinolysin and thrombin were demonstrated. The surprising finding of some thrombin here raises some new practical questions. Our suggested explanation is that some thrombin formation in artificially treated plasmas may not be detected, yet result in a "metathrombin" (combination of thrombin with natural antithrombin) from which thrombic activity is recoverable under certain circumstances, *e.g.*, following treatment with chloroform. Many questions as to the state of both prothrombin and thrombin in plasma and in serum are opened up by these new facts and some special studies on these matters are being pursued and have already been mentioned in a preliminary communication (19).

On casual observation, our PTP factor might appear to be acting like the so-called "accelerator" agents, two and possibly more types of which have occupied the attention of a number of recent investigators. A recent paper (20) in this JOURNAL adequately reviews the current status of these factors and particularly gives experimental evidence that Seegers' "serum accelerator globulin" preparation contains *both* Alexander's SPCA ("serum prothrombin conversion accelerator") *and* "labile factor." As we have mentioned earlier, the routine addition of a reasonable amount of serum AcG in our test systems should, we believe, ensure a sufficiency of these "accelerator" factors and make it unnecessary to enter into any discussion of them in the present connection. Rather, we may proceed to consideration of our results in terms of the "thromboplastic" problem.

This does bring the present work into relation with some other studies in the coagulation literature, particularly those which give attention to the idea of a thromboplastin precursor or "prothrombokinase" (Collingwood and MacMahon, 1912; Widenbauer, 1943) requiring activation in order to provide the "thromboplastic" (thrombokinase) factor for the clotting mechanism. In a series of recent papers Milstone reviews this literature and presents his new experimental data. His first paper appeared while our present studies were nearing completion and offered evidence of the enhancement of prothrombin conversion to thrombin in the presence of platelets when a (crude) plasma "globulin" preparation was added. In discussion Milstone (21) states: "Conceivably, platelets might contain prothrombokinase as suggested by Collingwood and MacMahon, or a platelet enzyme might activate the prothrombokinase ('thromboplastinogen') of plasma as suggested by Quick (1947). The present experiments do not decide between these hypotheses, nor do they eliminate other possibilities." Milstone's second paper (22) contributes some new technics for analyzing the mechanisms of blood coagulation into three phases. A "prothrombokinase" preparation from a euglobulin precipitate of bovine plasma served for the study of *plasma* thrombokinase (thromboplastin) formation, with the aid of calcium. A noteworthy point in discussion is . . . "thrombin does not catalyze the activation of prothrombin; but its possible effect on the activation of prothrombokinase remains undetermined." Milstone (23) prepared from the $BaSO_4$ adsorbate of bovine plasma globulins a material ("converter fraction") which . . . "hastened both the activation of prothrombin and the conversion of crude prothrombokinase. . . . The relation of . . . converter to thrombokinase activity resembles that reported for platelets plus 'globulin' (21)." In a still more recent paper (24) appearing after this present work was submitted for publication, Milstone reinvestigated the experimental activation of purified prothrombin by platelets, brain thromboplastin, lipoid thromboplastin, and crystalline trypsin, and emphasized the ability of the enzyme to act (*a*) alone, (*b*) as potentiator of lipid thromboplastin, and (*c*) together with platelets (even when amounts of trypsin and platelets were selected neither of which was demonstrably active alone). There

are obviously many points of confirmation and parallel interest between Milstone's work and ours, both the present and previously published (4). However, these serve only to emphasize the complexities of the problem and the final solution must await much future investigation.

CONCLUSIONS

1. Crytalline trypsin and "purified" serum fibrinolysin accelerate clotting of ordinary recalcified plasma and platelet-free plasma obtained with the silicone technic.

2. These enzymes accelerate and increase thrombin yields from heat-defibrinated plasma or other crude prothrombins.

3. They have no significant effects on the thrombin-fibrinogen reaction (apart from lytic interference) nor upon the activation of purified prothrombin with Ca and AcG but negligible thromboplastin.

4. They markedly potentiate platelet thromboplastic action.

5. *Well washed* platelets are thromboplastic, but negligibly AcG-like, and nonfibrinoplastic.

6. The platelet thromboplastin potentiating (PTP) effect of fibrinolysin shows an optimal enzyme concentration and lessens roughly parallel to weakening of lytic potency by dilution or by thermal deterioration.

7. It is, however, not abolished by effective antiproteases (antilytic) from pancreas (PI) and serum (antifibrinolysin), and only incompletely by soybean inhibitor (SBI), which, however, is antithromboplastic.

8. The PTP effects of trypsin, on the other hand, are completely abolished by the antilytic agents.

9. Very small amounts of thrombin (down to a limit) also have a PTP action, but the fibrinolysin used was practically thrombin-free.

10. A crude plasma protein fraction obtained at 30–40% $(NH_4)_2SO_4$ saturation of "prothrombin-free" (Seitz-filtered, × 5) plasma, when treated with $CHCl_3$, had PTP effects but contained some thrombin as well as fibrinolysin.

11. While many of these data support Ferguson's theory (1) of a clot-aiding role of "thromboplastic enzymes," perhaps still valid for trypsin, the inhibitor data controvert this for fibrinolysin. After considering the possible significance of thrombin, there remains the possibility of a new PTP factor, possibly the "prothrombokinase converter factor" postulated by Milstone (*op. cit.*), but apparently unrelated to the "accelerator" factors about which much work has appeared in the recent coagulation literature.

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Penicillin-Resistant Bronchopneumonia

TREATED WITH TERRAMYCIN[1]

S. A., Male, age 8

History Illness of 10 days' duration; temp. 104.8° F.; previous treatment with penicillin without response.

Therapy Terramycin, 1:8 Gm. daily for 8 days, 3.0 Gm. daily for 4 days; orally in divided doses q. 4 h.

Result Temperature started to fall in 24 hours; response described as "good".

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Terramycin

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New council-accepted broad-spectrum antibiotic orally effective—well tolerated

1. Terramycin may be highly effective even when other antibiotics fail.[1]
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Suggested for: *acute pneumococcal infections, including lobar pneumonia, bacteremia; acute streptococcal infections, including erysipelas, septic sore throat, tonsillitis; acute staphylococcal infections; bacillary infections, including anthrax; urinary tract infections due to E. coli, A. aerogenes, Staphylococcus albus or aureus, and other Terramycin-sensitive organisms; acute brucellosis (abortus, melitensis, suis); hemophilus infections; acute gonococcal infections; lymphogranuloma venereum; granuloma inguinale; primary atypical pneumonia; typhus (murine, epidemic, scrub); rickettsialpox.*

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1. King, E. Q.; Lewis, C. N.; Welch, H.; Clark, E. A., Jr.; Johnson, J. B.; Lyons, J. B.; Scott, R. B., and Cornely, P. B.: J.A.M.A. 143:1 (May 6) 1950.
2. Herrell, W. E.; Heilman, F. R.; Weltman, W. E., and Bartholomew, L. A.: Proc. Staff Meet. Mayo Clin. 25:183 (April 12) 1950.

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